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EARLY METABOLIC DERANGEMENTS IN ACQUIRED OBESITY

A STUDY OF YOUNG OBESITY-DISCORDANT MONOZYGOTIC TWINS

Sanna Kaye

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To My Family

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LIST OF ORIGINAL PUBLICATIONS

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I Kaye S*, Pietiläinen KH*, Kotronen A, Joutsi-Korhonen L, Kaprio J, Yki-Järvinen H, Lassila R†, Rissanen A†
Obesity-related derangements in coagulation and fibrinolysis: a study of obesity-discordant monozygotic twins
Obesity (Silver Spring), 2012 Jan;20(1):88-94

II Kaye S, Maranghi M, Bogl L, Taskinen M-R, Kaprio J, Rissanen A, Pietiläinen K
Acquired liver fat is a key determinant of serum lipid alterations in healthy monozygotic twins
Obesity (Silver Spring), 2013 Sep;21(9):1815-22

III Bogl LH*, Kaye S*, Rämö J, Kangas AJ, Soininen P, Hakkarainen A, Lundbom J, Lundbom N, Ortega-Alonso A, Rissanen A, Ala-Korpela M, Kaprio J, Pietiläinen KH
Abdominal obesity and circulating metabolites: a twin study approach
Metabolism. 2016 Mar;65(3):111-21

IV Kaye S, Lokki A-I, Hanttu A, Nissilä E, Heinonen S, Hakkarainen A Lundbom J, Lundbom N, Tyyninen O, Saarinen L, Muniandy M, Rissanen A, Kaprio J, Meri S, Pietiläinen KH
Upregulation of Early and Downregulation of Terminal Pathway Complement Genes in Subcutaneous Adipose Tissue and Adipocytes in Acquired Obesity
Front Immunol. 2017 May 16;8:545. doi: 10.3389/fimmu.2017.00545. eCollection 2017

The publications are referred to in the text by their roman numerals.
*shared first authorship
†shared contribution

ABBREVIATIONS

Δ	intra-pair difference
AT	Adipose Tissue
ApoA1	Apolipoprotein A1
ApoB	Apolipoprotein B
BMI	Body Mass Index
BCAA	Branched-Chain Amino Acids
CLS	Crown-like Structure
CVD	Cardiovascular disease
DEXA	Dual X-ray Absorptiometry
DNL	De Novo Lipogenesis
DZ	Dizygotic
C	Cholesterol
C3a	Complement component 3a
CVD	Cardiovascular Disease
F	Coagulation Factor
FA	Fatty Acid
FFA	Free Fatty Acid
HDL	High Density Lipoprotein
hsCRP	High-sensitivity C-Reactive Protein
HOMA	Homeostatic model assessment – a measure of insulin-resistance
Ia	Intra-abdominal
IDL	Intermediate Density Lipoprotein
IR	Insulin resistance
Lf	Liver fat
MAC	Membrane attack complex
M-value	Measure of insulin sensitivity
MetS	Metabolic syndrome
MZ	Monozygotic
NAFLD	Non-Alcoholic Fatty Liver Disease
NMR	Nuclear Magnetic Resonance
PAI-1	Plasminogen Activator Inhibitor-1
PUFA	Polyunsaturated Fatty Acids
Sc	Subcutaneous
SD	Standard Deviation
SE	Standard Error
T2D	Type 2 Diabetes
TG	Triglyceride
VLDL	Very Low Density Lipoprotein
WC	Waist circumference

ABSTRACT

Background The rate of obesity is increasing with an alarming rate worldwide. Obesity increases the risk of diabetes or atherosclerotic cardiovascular disease. Additionally, obesity associates with co-morbidities such as hypertension, non-alcoholic fatty liver disease predisposing to steatohepatitis, blood thrombosis, and incidence of certain cancers. Thus, obesity is posing a significant threat to public health. Several genes control body weight and the development of obesity-related metabolic complications. Certain genetic variants have been identified that predispose to the risk of developing obesity and obesity-related diseases. However, environmental or acquired factors may modify gene function and unique individual habits may contribute to the development or outcome of metabolic disturbances. In population-based studies on obesity-related metabolic derangements, it is impossible to disentangle the contribution of genes from unique environmental factors. Nonetheless, monozygotic (MZ) twins carry identical genes. The comparison of MZ and dizygotic (DZ) twin pairs with discordance in obesity can help to elucidate the magnitude of acquired or environmental factors. In obesity, the underlying molecular mechanisms are complex with intertwining pathophysiology of metabolic pathways in different organ systems. Thus, there is a need for improved understanding regarding the role of adipose tissue distribution and function underlying the metabolic risk factors.

Aims This thesis employs biomarker analyses to: 1) characterise the derangements in blood coagulation and fibrinolysis in acquired obesity; 2) capture the magnitude of alterations in lipid metabolism, serum lipoprotein particle profile, and its quality and quantity in obesity; 3) examine the contribution of genetic and environmental factors impacting the association of abdominal obesity and serum NMR metabolites; and 4) describe the differences in the activation of the complement system between obesity-discordant MZ co-twins by analysing the plasma levels and the expression of the complement-system-related genes in subcutaneous fat and isolated adipocytes.

Materials and methods The study subjects derive from two population-based cohorts of young Finnish twins – FinnTwin12 and FinnTwin16. The TwinFat cohort comprises 286 monozygotic (MZ) and dizygotic (DZ) twin individuals. The rare cohort of obesity-discordant (Body mass index (BMI) -difference > 3kg/m^2) MZ twin pairs (n=14-26 pairs, age 23–36) underwent an intensive study protocol. Their control group comprised obesity-concordant (within-pair BMI- difference < 3kg/m^2) MZ twin pairs. The genetic and environmental contributions to the association between metabolism and abdominal obesity were determined from a larger group comprising MZ and DZ twins (n=1368) from FinnTwin cohort. The body fat mass and distribution was assessed with Dual-Energy X-ray Absorptiometry (DEXA), magnetic resonance imaging (MRI), and proton magnetic spectroscopy to determine the liver fat content. Several biomarkers (lipid and glucose metabolism, inflammation, NMR metabolites) were measured from plasma. The expression of complement system-related genes was assessed with microarray from subcutaneous adipose tissue (AT) biopsies. Data on the habitual physical exercise and eating habits was collected using questionnaires. By comparing BMI-discordant MZ co-twins, it is possible to adjust for genetic background.

Results Several metabolic derangements predisposing to cardiovascular diseases and diabetes emerged in acquired obesity. Obese co-twins of BMI-discordant pairs presented elevated blood coagulation activity and increased levels of fibrinogen and the activities of factors (F) IX, XI, XII, as well as plasminogen activator inhibitor-1 (PAI-1), a marker of

fibrinolysis. The lipoprotein particle number increased in acquired obesity. The most pronounced difference was the increase in pro-atherogenic LDL-particles and triglyceride-rich VLDL particles. In contrast, the number of HDL particles and the amount of HDL cholesterol was reduced. The composition of the lipoprotein particles remained unchanged in obesity. The pro-atherogenic alterations in lipoprotein profile emerged only if obesity was accompanied by concomitant increase in liver fat. Both genetic and environmental factors contributed to the association between unhealthy NMR lipoprotein and metabolite profile and abdominal obesity. Shared genes were found to determine the co-occurrence of these phenotypic traits. Additionally, the levels of branched-chain amino acids, α1-glycoprotein, lactate, and urea were elevated in acquired obesity and associated with hyperinsulinemia. The activation of the complement system associated to low-grade inflammation in subcutaneous (sc) adipose tissue (AT). In acquired obesity, the expression of several complement system related genes in sc AT was altered and plasma concentration of C3a was increased. Measures of coagulation, lipoproteins, NMR metabolite profile, and complement-system activity were similar between the obesity-concordant twin pairs.

Conclusions Widespread derangements in metabolism emerge predisposing to the increased risk for morbidity in young healthy adults in acquired obesity. The fatty liver is a key player behind alterations in lipoprotein metabolism. Increased adiposity alone does not define the metabolic risk; it is important to assess the risk by determining the fat distribution together with biomarker analyses. In this thesis, several new potentially useful biomarkers are presented to further characterise and define the metabolic risk in obese individuals. These biomarkers relate to blood coagulation, metabolism, and inflammation. Complement system activation associates to low-grade inflammation in AT, and may be important in protecting AT viability around the sites of necrotic cells. The results show that despite genetic predisposition, the individual's unique lifestyle significantly contributes to derangements in metabolism. Weight-management is crucial in primary prevention of metabolic morbidity.

TIIVISTELMÄ

Tausta Viime vuosikymmeninä lihavuus on yleistynyt huolestuttavasti ja on merkittävä kansanterveydellinen uhka. Lihavuus lisää riskiä sairastua diabetekseen ja valtimonkovettumatautiin. Lihavuus altistaa myös verenpainetaudille, ei-alkoholiperäisen rasvamaksan ja tätä kautta maksatulehduksen kehittymiselle ja veritulpan muodostumiselle. Lisäksi lihavuus liitetään useiden eri syöpäsairauksien ilmaantuvuuteen. Useat eri geenit säätelevät painoa ja lihavuuteen liittyvien aineenvaihdunnan häiriöiden kehittymistä. Sekä liitännäissairauksien että lihavuuden kehittymiselle altistavia perintötekijöitä on tunnistettu. Koska ympäristötekijät voivat säädellä geenien toimintaa, elämäntavat voivat vaikuttaa sairastumisriskiin. Väestöpohjaisessa aineistossa ei voida erottaa geenien ja ympäristön osuutta aineenvaihduntahäiriön taustalla. Koska samanmunaisilla (monotsygoottiset, MZ) kaksosilla on sama geeniperimä, eripainoisia MZ-kaksosia vertailemalla voidaan vakioida perimän vaikutus ja kuvata hankitun lihavuuden merkitystä aineenvaihduntamuutosten taustalla. Lihavuuteen liittyvien aineenvaihdunnan häiriöiden mekanismit ovat monimutkaisia, ja nämä vaikuttavat monien patofysiologisten kokonaisuuksien ja elinjärjestelmien kautta. Rasvakudoksen sijainnin ja ominaisuuksien merkitys aineenvaihduntahäiriöiden taustalla on keskeinen ja osittain vielä puutteellisesti tunnettu, joten näitä seikkoja on syytä tutkia lisää.

Tavoitteet Tutkimme eripainoisilla identtisillä kaksospareilla biomarkkereita apuna käyttäen 1) muutoksia veren hyytymistä ja liukoisuutta säätelevissä tekijöissä; 2) minkä verran muutoksia nähdään veren rasvojen ja lipoproteiinien määrässä ja koostumuksessa; 3) perimän ja ympäristön osuutta erikseen vyötärölihavuuden ja aineenvaihdunnan muutosten taustalla; 4) komplementtijärjestelmän aktivaatiota ja järjestelmän osakomponenttien geenien ilmentymistä ihonalaisrasvakudoksessa.

Materiaali ja menetelmät Tutkimusaineisto pohjautuu FinnTwin12 ja FinnTwin16 – väestökohortteihin. TwinFat- aineisto sisältää yhteensä 286 MZ- ja DZ-kaksosta. TwinFat-aineistosta kutsuttiin kattaviin aineenvaihduntatutkimukseen eripainoisia (painoindeksin (BMI) ero parien välillä > 3kg/m^2) MZ kaksospareja (n=14-26 paria, ikä 23–36 vuotta) ja heidän vertailuryhmänään oli samanpainoisia (BMI-ero < 3kg/m^2) MZ kaksospareja (n=9-14 paria). Tutkittaessa geenien ja ympäristön välistä vuorovaikutusta vyötärölihavuuteen liittyvän aineenvaihduntamuutosten taustalla, hyödynnettiin laajempaa kaksosaineistoa (n=1358), jossa mukana oli myös ei-identtisiä kaksospareja. Kehon rasvan määrä ja jakauma määritettiin kuvantamismenetelmin (DEXA, magneettikuvaus, magneettispektroskopia). Aineenvaihduntamarkkereita (sokeri- ja rasva-aineenvaihdunta, inflammaatio, komplementtijärjestelmän aktiivisuus) määritettiin paastoverinäytteestä. Ihonalaisrasvasta otetusta kudosbiopsiasta määritettiin komplementtijärjestelmään liittyvien geenien ilmentymistä. Kyselylomakkeilla kerättiin tietoa kaksosparien liikunta- ja ravitsemustottumuksista.

Tulokset Hankittu lihavuus toi esiin merkittäviä verisuonitukokselle, valtimokovettumataudin sekä diabeteksen kehittymiselle altistavia aineenvaihduntamuutoksia. Lihavilla kaksosparikeilla plasman fibrinogeenin pitoisuus lisääntyy sekä veren hyytymistekijöistä faktori (F) IX :n, XI:n, XII:n ja veritulpan liukenemiseen liittyvän PAI-1:n aktiivisuus kohoaa. Lihavuudessa veren lipoproteiinipartikkeleista erityisesti aterogeenisten LDL-partikkeleiden ja triglyseridipitoisten VLDL-partikkeleiden määrä lisääntyy ja HDL-partikkeleiden ja –kolesterolin määrä pienentyy. Lipoproteiinipartikkeleiden koostumus ei muutu

lihavuudessa. Valtimokovettumataudille altistavia muutoksia lipoproteiinipitoisuuksissa havaittiin vain, mikäli lihavuuteen liittyi maksan rasvoittumista. Sekä perimä että ympäristötekijät vaikuttavat osaltaan terveydelle epäedullisen NMR-metaboliittiprofiilin ja vyötärölihavuuden väliseen yhteyteen. Näiden piirteiden ilmeneminen on osittain samojen geenien säätelemää. Hankitun lihavuuden vaikutuksesta haaraketjuisten aminohappojen, laktaatin, urean ja α1-glykoproteiinin pitoisuus nousee. Merkkinä komplementtijärjestelmän aktivaatiosta lihavuudessa järjestelmään liittyvien geenien ilmentyminen ihonalaisrasvakudoksessa muuttuu ja plasman C3a-komponentin pitoisuus lisääntyy. Kehonkoostumuksessa, aineenvaihduntaa kuvaavien biomarkkereiden pitoisuuksissa tai komplementtijärjestelmän aktivaatiossa ei havaittu eroja samanpainoisten MZ kaksosparikkien välillä.

Yhteenveto Hankittu lihavuus toi esiin useita sairauksille altistavia aineenvaihduntamuutoksia jo varhain nuorilla terveillä kaksosilla. Maksan rasvoittumisella on keskeinen merkitys erityisesti rasva-aineenvaihdunnan häiriön synnyssä. Aineenvaihduntahäiriöiden riski lihavuudessa on yksilöllinen ja tähän vaikuttaa oleellisesti kehon rasvan jakauma. Väitöskirjassani nousee esille uusia veren hyytymisaktiivisuutta, aineenvaihduntaa ja inflammaatiota kuvaavia biomarkkereita, joita voidaan mahdollisesti käyttää arvioitaessa lisääntynyttä sairastuvuusriskiä lihavuudessa. Komplementtijärjestelmän aktivaatio liittyy matala-asteiseen tulehdukseen rasvakudoksessa, mutta tämä voi myös olla keskeinen tekijä suojaamaan nekroottisten alueiden ympäristöä kudostuholta. Väitöskirjatutkimuksen tulokset osoittavat, että perimästä riippumatta elintavoilla on merkitystä aineenvaihduntamuutosten synnyssä. Sairastumisriskin ennaltaehkäisyssä painonhallinnalla on keskeinen rooli.

1. INTRODUCTION

The prevalence of obesity has almost doubled since 1980, and obesity is now considered to be a global epidemic. According to the World Health Organization (WHO), over 39% of adults are overweight (Body Mass Index (BMI) > 25kg/m^2), and over 13% of adults are obese (BMI > 30kg/m^2) (1). In Finland, the prevalence of obesity follows global trends. The incidence of obesity temporarily plateaued among adults in early 21st century, but the current rate of obesity is still increasing (2)(3)(4). Obesity is common in both adults and children (5)(6), and this may limit children's future health and life expectancy as adults (7). Overweight and obesity associate with increased risk of mortality and morbidity in adulthood creating a burden to the global economy. An estimated 7.7% of all deaths in the European Union in 1997 were attributable to obesity: 70% of those were due to cardiovascular disease (CVD) and 20% due to cancers (8). Even after taking into consideration the hazardous effect of smoking, excess weight contributes significantly to the risk of death in middle age subjects (9)(10). Therefore, one of the key global public health targets is the prevention of obesity.

Following the growing rate of obese subjects, the number of the patients with type 2 diabetes (T2D), characterised by chronically elevated plasma glucose levels, has globally escalated from 108 million to 422 million since 1980 (11). The risk of developing diabetes is 30% higher in overweight, and up to 90% higher in very obese, compared to normal weight subjects (9). Diabetes contributes significantly to the shorter life expectancy in obesity: the overall mortality in diabetes is higher compared to the healthy population (12)(13). Like in obesity, CVD is the predominant cause of death in diabetes.

Dyslipidemia is a key pillar in the pathogenesis of CVD. Overweight and obese subjects, as well as the patients with metabolic syndrome, often present pro-atherogenic lipid profile predisposing to CVD (14)(15)(16). As the prevalence of the diabetes correlates strongly and positively to degree of the obesity, the prevalence of dyslipidemia in obese subjects varies. The pro-atherogenic lipid profile associates particularly to abdominal obesity (17)(18). Therefore, not only the body size, but the body composition, is an important measure when assessing the excess risk for CVD (19). Proatherogenicity typically associates with abdominal obesity and fatty liver. In obesity, the clustering of the CVD risk factors is common. Derangements in glucose and lipid metabolism combined with arterial stiffness and hypertension is a common co-morbidity characterising metabolic syndrome (MetS) (Figure 1). In addition, obesity increases the risk of venous thrombosis (20), gastrointestinal tract diseases including non-alcoholic fatty liver disease (NAFLD) (21), and certain cancers (22). Obstructive sleep apnea, a mechanical obstruction of upper airways predisposing to hypoxic periods during sleep associates with metabolic stress, and may further increase cardiovascular risk in obesity (23). Excess weight puts mechanical load on joints in the lower back and limbs, and degenerative osteoarthritis is higher in obese than normal-weight subjects (24). It is noteworthy that wellbeing and quality of life, may be prematurely compromised in obesity (25). The link between depression and obesity is reciprocal (26).

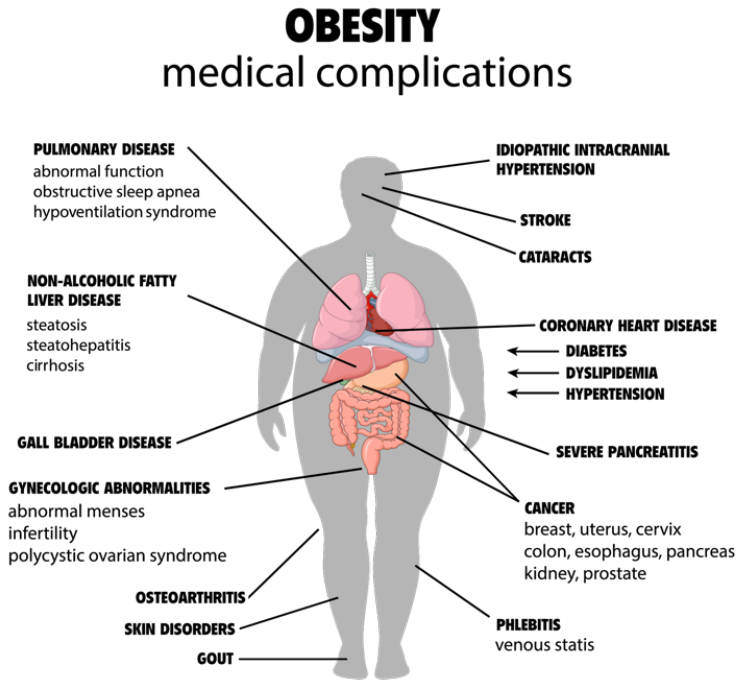

Figure 1 Medical complications in obesity

Approximately 40–70% of variation in body mass index (BMI) is attributable to genetic factors (27)(28)(29). At the individual level, the monogenic form of obesity is more severe, the genetic contribution to body weight at the population level is predominantly multifactorial (27). Clustering of obesity related co-morbidities suggests that the genetic pathways may be pleiotropic, thus the same set of genes regulate both the weight, and glucose and lipid metabolism (30). Certain subjects develop obesity-related metabolic derangements earlier than others, potentially due to genetic predisposition. There is evidence that environmental influences can modify the outcome or the severity of phenotype (31)(32). For example, type 2 diabetes is heritable (30)(33), but as described earlier, obesity correlates strongly with the prevalence of diabetes. Environmental factors, such as eating habits leading to positive energy balance and obesity in subjects genetically vulnerable to develop diabetes, are extremely disadvantageous and may lead to the earlier development or more rapid progression of the clinical disease.

Population-based epidemiological studies on obesity cannot answer the question of how much of the known association between obesity and metabolic derangements is due to the genetic factors, and how much of the variation is due to the unique environment. Twin studies are the key when dissecting the effect of the genetic factors from the effects of the environment. A rare cohort of Finnish obesity-discordant monozygotic (MZ) twins has elucidated these questions (34)(35)(36)(37). The case-control comparison of MZ co-twins discordant on weight is ideal to reveal the magnitude of the unique environmental factors, as their genetic background is shared. This thesis focuses on describing the impacts that acquired obesity and differences in body composition have on metabolism. The results complement the efforts of 15 years of data collection and earlier studies in the same cohort (36). The results disclose previously uncharacterised similarities and differences in blood coagulation and fibrinolysis, lipoprotein metabolism and the complement-system activity within obesity-discordant pairs. This twin cohort is young and examined before clinical

manifestation of clinical diseases, the results provide new insights into the role of the adipose tissue (AT) at the molecular level in the pathogenesis of obesity-related co-morbidity.

2. THE REVIEW OF THE LITERATURE

2.1 Body composition

Subcutaneous (sc) fat is the largest adipose tissue (AT) depot. Lesser amounts of AT localise viscerally (visceral adipose tissue, VAT) in the intra-abdominal (ia) cavity surrounding large vessels and organs (38). Sc fat that is distributed to the pelvic or thigh area is also referred to as 'gynoid fat', whereas AT localised proximally in the trunk, waist, or thoracic area is called 'android fat' (38)(39). Gender (40)(41), age (42), and ethnicity (43)(44) are important determinants of body fat distribution. Women have more fat mass than men who have a higher proportion of lean mass on total body mass due to higher muscle mass (45). The gynoid fat distribution is typical for premenopausal women. In comparison to men, women have a lower VAT to total-body-fat ratio even when the overall total-body-fat percentage has been accounted for (40). Body fat percentage increases with age and this is partly due to the loss of muscle mass (sarcopenia) (46) and alterations in sex steroids (42). Upon aging, maintenance of muscle mass associates with metabolic health. If sarcopenia occurs, obesity metabolism may be compromised in comparison to normal-weight individuals with low muscle mass (46). After estrogen levels decrease in menopause, the proportion of ia fat also increases in women (47). In men, low testosterone levels associate with larger amounts of VAT (48)(49). In addition, the accumulation of ia fat is characteristic to pathological endocrine conditions, such as hypercortisolism (Cushing's syndrome) and genetic or acquired lipodystrophies (50). Body mass index (BMI, kg/m^2) correlates well with overall adiposity, especially in high BMI ranges (42). Waist circumference (WC) or waist-to-hip ratio (WHR) correlate well with the amount of VAT, thus they complement BMI in the assessment of adiposity and body fat distribution.

2.1.1 Visceral fat distribution associates to the pathophysiology of diabetes and CVD

Body fat distribution is an important predictor of derangements in systemic metabolism. In particular, VAT associates with an adverse metabolic phenotype (51). The amount of VAT associates strongly positively with alterations in glucose and lipid metabolism, hyperglycaemia, and hypertriglyceridemia, as well as elevated blood pressure (51)(52) predisposing to the risk of CVD events. The relationship between VAT and metabolic status is pivotal, as lower ratio of VAT to total adiposity and sc fat, and larger relative amounts of femoral fat, associate with healthier glucose and lipid metabolism (53)(54). In development of metabolic derangements, however, the role of sc fat, the largest body fat depot, cannot be disregarded. High BMI with excess sc fat mass associates with significant co-morbidity. The correlation between the amount of sc fat and metabolic risk measures occurs, but it is not linear (55).

2.1.2 Body composition is determined by genes and modifiable environmental factors

Both body size and composition are genetically controlled. The genetic contribution to height is as high as 80% (56). The heritability of BMI is 40–70% depending on the cohort. Monogenic obesity, due to mutations of a single gene, is rare. Characteristic to monogenic

obesity is the onset of obesity occurring already in early childhood. It is often syndromic associating with comorbidity and developmental deficits (57). Recent advances in understanding the human genome from large population-based cohorts have brought new insight in obesity research and elucidated the genetic landscape in common obesity. Numerous genetic loci associate with BMI suggesting the genetic background of obesity is polygenic (58). The impact of genetic variants on weight varies with age, the effect being largest during childhood (59). When combining the effects of the known genetic variants associating with BMI, it is estimated that the net effect of genes explains over 20% of the variation in BMI at the population level.

Bouchard et al (60) showed in a pilot twin study that an increase in the amount of VAT did not correlate with gained total fat mass. The variance in gaining VAT was significantly higher between the pairs than within MZ twin pairs. Thus, the genetic contribution determines the inter-individual variation also in the amount of VAT (60). Recent genome studies have supported this finding. Several genetic variants have been associated to determine body fat distribution (50) and also the lean mass (61). The genetic loci associating to WHR have been shown to present greater effect in women in comparison to men (62).

Insight on the impact of diet or exercise on body size and composition derive from randomised and controlled lifestyle intervention studies. The imbalance in energy intake in relation to overall energy consumption leads to weight gain. Positive energy balance and excess calories increase both body lean mass and adiposity, but the nutritional content of the diet may also influence the changes in body composition. Sugar-sweetened beverages and fructose have been shown to favour ectopic and visceral fat accumulation (63)(64). In addition, dietary fatty acid composition modifies body fat distribution as the indigestion of saturated fats may associate with VAT accumulation (65), whereas mono-unsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) may prevent the gain of VAT and associate with lower WC (65)(66). Exercise is an effective way to consume energy and control body weight, with positive effects on body composition. Aerobic exercise effectively reduces visceral fat regardless of the intensity and the volume of the exercise regime (67)(68). Additionally, exercise increases lean mass, bone mass, and mineral density (69). Anaerobic resistance training, in particular, associates to increase in muscle mass (70). Both anaerobic and aerobic exercise have health benefits and are shown to reduce the risk of CVD in high risk individuals (71).

2.1.3 Regulation of appetite

The sense of hunger and satiety facilitate controlling the portion size upon meal time and not eating too frequently. Incretin hormones, such as glucagon-like peptide (GLP-1), cholecystokinin (CCK), peptide tyrosine-tyrosine (PYY), glucose-dependent insulin releasing polypeptide (GIP), and ghrelin from the upper gastrointestinal tract contribute to the sensation of satiety or hunger (72). In obesity, the effect of incretins in the regulation of food intake is blunted (73). The lack of the function of incretin hormones weakens the satiety signals in brain. Increased appetite leads to hyperphagia and a surplus of indigested calories (74). This leads to positive energy balance if the energy expenditure does not increase following increased calorie intake.

AT is the largest endocrine organ secreting adipokines, adipose tissue derived hormones. Leptin, the most documented adipokine, regulates appetite via the hypothalamus by

monitoring satiety signals (75). The plasma levels of leptin correlate with the amount of AT and the levels raise in obesity (76)(77). However, in obesity, leptin signalling in the brain is defective and the appetite is not adequately suppressed. Similarly with ghrelin, in obesity the ghrelin levels are not suppressed after a meal and flattened levels may contribute to the tendency of snacking (72). In conclusion, in obesity, rather than increased absorption of nutrients, hyperphagia contributes to weight gain.

2.2 Adipose tissue morphology

White adipocytes containing triglyceride droplets are the largest cells in AT. The turnover of mature adipocytes is 8–10% per year in humans, and new cells are recruited from precursors to replace the dying adipocytes (78)(79). Adipose tissue stroma is cell rich and comprises extracellular matrix and immune cells. Adipocytes in AT are surrounded by vasculature that supplies oxygen, nutrients, and other blood components (80). When energy balance is positive, AT needs to expand to store energy. The cell size of the adipocytes grows and concomitantly, new adipocytes are recruited from precursors for storing excess energy (80). Thus, AT expansion is due to both cell hyperplasia and hypertrophy. AT vessels contribute to adipose tissue expansion providing adipocyte progenitor cells from blood flow (81). In healthy AT, the vasculature grows allowing the expansion and remodelling of AT (78)(82).

Certain adipocytes have distinct features in energy dispersion. Mitochondria-rich adipocytes have a brown appearance. Brown adipocytes are distinguishable from white adipocytes expressing uncoupling-protein-1 (UCP-1)(80). Brown AT typically localises in paracervical and suprascapular regions in adults (83). Characteristic to brown AT is the dispersion of energy as heat. Beige adipocytes are cells are predominantly comparable to white adipocytes but by beta-adrenergic stimulation they express UCP-1 and functional characteristics resemble those of brown adipocytes (84).

Like body size, AT morphology is also under genetic control. Sc AT cellularity within MZ co-twins is similar (85)(86). Similarly, weight loss does not have a significant effect on overall cellularity in obese subjects (80)(87). In acquired obesity, sc AT is hypoplastic and adipocytes are hypertrophic. AT adipocytes grow in size, but adipocyte number does not increase (86). Obesity-discordant MZ co-twin comparison confirmed that these alterations emerge independent of genetic contribution (86). Obese subjects with hypoplastic sc AT presented features of unhealthy metabolism characterised by hyperinsulinemia, hyperglycaemia, and pro-atherogenic lipid profile (86).

2.3 Characteristic of healthy adipose tissue

In functional and healthy AT, in a state of overnutrition, energy metabolism favours de novo lipogenesis and lipid storage in adipocytes (88). De novo lipogenesis (DNL) is a physiological way to dispose glucose in AT. Free fatty acids (FFAs) deriving from diet or via de novo lipogenesis from glucose are esterified into triglycerides (TGs), then processed in the endoplasmic reticulum to form lipid droplets within adipocytes (78). In AT, insulin promotes glucose and lipid uptake and suppresses lipolysis, the net effect is the promotion of nutrient storage. The nutrient storage in sc AT adipocytes associates with the maintenance of normoglycemic plasma glucose levels and systemic insulin sensitivity (89)(90). Adipocytes release energy from TG droplets in demand. The effect of catecholamines and glucagon in AT is opposite to that of insulin releasing energy in

demand. In lipolysis, the hormone sensitive lipase (HSL) hydrolyses TGs back to glycerol and FFAs. Insulin inhibits HSL and thereby AT lipolysis.

Functional insulin signalling is essential for storing nutrients efficiently in AT and maintaining tissue integrity (78). Peroxisome proliferator-activated receptor gamma (PPARγ) is a strong insulin-sensitizer in AT (91) (92). It induces the expression of transmembranic GLUT4 receptors thereby facilitates glucose uptake and providing substrate for DNL (93). Fatty acid transport proteins facilitate fatty acid storage as well as controlling lipolysis (94). Carbohydrate-responsive-element-binding protein (ChREBP) is a regulator of lipogenic and glycolytic genes (89)(95). It regulates PPARγ activity and advances glucose disposal in AT by increasing DNL (95)(89). Lipoprotein lipase (LPL) activity is crucial for TG storage in AT. The enzymatic activity of LPL is insulin-dependent (96). The high activity of LPL is protective against metabolic derangements in obesity (97). Pivotally, in obesity and insulin resistance (IR), the suppressed LPL activity in adipose tissue may impair TG storing (97)(98).

2.3.1 AT mitochondria

Glucose disposal and fatty acid storage require energy and the mitochondria in adipocytes are responsible for oxidative energy supply in AT (99). The beta-oxidation of FFAs takes place in adipocyte mitochondria when FFAs are oxidised to acetyl-co-enzyme-A, and subsequently to adenosine di-phosphate (ADP) and reduced to nicotinamide adenine diphosphate (NADH) providing energy for metabolism (78). Ketone bodies are the end products of beta-oxidation transporting energy to other tissues from AT(100). AT can increase its energy production capacity during adipocyte differentiation by recruiting new adipocytes and thus increase the total number of adipocytes. Concomitantly, the biogenesis of mitochondria and subsequent increase in beta-oxidation capacity are characteristic to healthy AT and tissue remodelling in overnutrition (78). If mitochondrial oxidative phosphorylation capacity is compromised, the resultant metabolic effects are widespread. Diminished oxidative capacity leads to increase in intracellular stress and upregulation of proinflammatory signals, eventually leading to cell death. Areas of dying cells generate proinflammatory milieu and impair insulin signalling in AT. Mitochondrial dysfunction associates with the development of systemic insulin resistance and diabetes (101) and also precedes the manifestation of clinical disease (102)(103)(104). The reduced mitochondrial DNA copy number and the down-regulation of the mitochondrial gene-expression in AT has been demonstrated in acquired obesity (104).

2.4 The role of subcutaneous AT in development of the insulin resistance and lipotoxicity

In IR obesity, fatty acid storage and glucose uptake in AT are defective (Figure 2). The function of the key enzymes of these processes, including GLUT4, are suppressed (89)(105). Concomitantly, when insulin fails to suppress lipolysis, FFAs spill from AT, in hyperglycaemic and IR conditions HSL levels increase accelerating lipolysis (106). The lipolysis in sc AT, which is the largest adipose depot, contributes significantly to the circulating FFA levels (107). The AT lipolysis plays an important role in the pathogenesis of insulin resistance as surplus of FFAs leads to accumulation of TG droplets to ectopic tissues, such as to the liver, the heart, or muscle. Release of FFAs from AT and lipid accumulation ectopically eventually impair insulin sensitivity systemically. Thus, the consequences of lipid spillover from dysfunctional AT are widespread (108). The amount

of VAT and abdominal obesity correlate with ectopic lipid accumulation (109). Therapeutic obesity intervention by bariatric surgery increases the activity of the key enzymes of DNL facilitating energy storage in sc AT(89).

The IR obesity is characterised by impaired glucose tolerance as higher insulin levels are needed for glucose uptake to the tissues to maintain normoglycaemia. Glucose absorption is accelerated postprandially in obese subjects. As a physiological response to indigested glucose, insulin levels rise rapidly. In obesity, insulin secretion is delayed due to reduced secretion of incretin hormones from the upper gastrointestinal tract (74). Incretins normally suppress glucagon secretion, but in obesity they fail to do so, and glucagon levels rise. As a result, hyperglucagonaemia contributes to the pathophysiology of hyperglycaemia by releasing more glucose from glycogen storage (110). Muscle glycogen synthesis and glucose oxidation for energy production normally accounts for most postprandial glucose disposal. In obesity, intramuscular IR resulting from intramuscular TG accumulation inhibits glucose uptake into myocytes and contributes to the pathophysiology of hyperglycaemia (111)(112). Adiponectin is a potent insulin-sensitising adipokine and its receptors are expressed in all tissues but predominantly in muscle and in liver (113). It ameliorates insulin resistance and has favourable effects on body weight (114). Adiponectin levels decrease in obesity and with age (113)(115). In obesity, pro-inflammatory adipokines, such as tumour necrosis alpha and interleukins, modify glucose tolerance and lipid metabolism. Low-grade inflammation links AT to the pathophysiology of CVD (116), diabetes (117), neurocognitive diseases (118), and carcinogenesis (118).

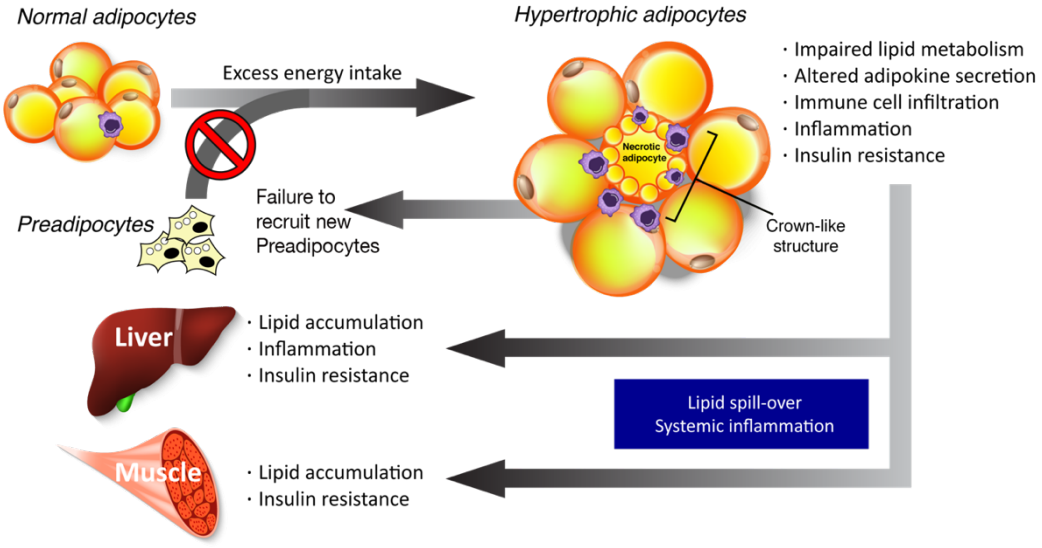

Figure 2 Features of unhealthy adipose tissue

In conclusion, to be able to expand, AT needs functional mitochondria for its energy production. If there is lack of oxidative capacity in the AT mitochondria and the recruitment of new adipocytes from precursor cells fail, AT is not able to store nutrients in sc AT depots. The accumulation of lipids in VAT and in ectopic tissues intracellular associates with IR. In obesity, the levels of insulin sensitising adipokines are reduced. Ectopic fat interacts with surrounding tissues in endocrine and paracrine manner. In the pancreas, TGs contribute to beta-cell failure and insulin secretion (119). The perivascular fat around coronary arteries

associates to coronary calcification, myocardial steatosis, and diastolic dysfunction in the absence of obstructive coronary artery disease (120).Thus, AT dysfunction leads to lipotoxicity due to TG accumulation in ectopic tissues and impaired insulin sensitivity. Ectopic fat and systemic low-grade inflammation are key contributors to the pathophysiological mechanisms of metabolic co-morbidity.

2.5 Non-alcoholic fatty liver disease (NAFLD) – a manifestation of ectopic adipose tissue

In obesity, the risk of developing non-alcoholic fatty liver disease (NAFLD) is 3.5-times higher compared to normal weight individuals (121). The global prevalence of NAFLD is estimated to be 25% (122) but it varies considerably, from 15% in paediatric up to 90% in the severely obese population (123). Elevated alanine transferase levels (ALT) > 30 U/L in men, and > 20 U/L in women associate with increased intrahepatic TG accumulation (124). In NAFLD, intrahepatic TG content exceeds 5% (124). The liver biopsy is a gold standard in NALFD diagnostics, but radiological screening has gained advantage being non-invasive. The ultrasound screens fatty liver with a sensitivity of 60–94% and specificity of 66–95% (125). Liver proton magnetic spectroscopy offers higher reproducibility compared to ultrasound, and its reliability has been validated against liver biopsies (125).

NAFLD is a manifestation of metabolic syndrome (MetS). The patients with MetS with NAFLD show a worse endothelium-dependent vasodilation compared with patients with MetS without NAFLD (126). NAFLD with liver IR leads to the failure of insulin to suppress gluconeogenesis in the liver resulting hyperglycaemia (127). Over 20% of the patients with NAFLD have T2D (122), and more than 2/3 of NAFLD patients are dyslipidemic (122)(128). Fatty liver promotes the progression of liver injury and increases the risk for non-alcoholic steatohepatitis (NASH) (129). Altered intracellular cholesterol homeostasis is involved in pathophysiological mechanisms in NASH (129). Chronic inflammation characterises NASH, and patients are at risk of progression of liver disease to fibrosis and liver cirrhosis, and irreversible loss of organ function (130). NASH also predisposes the development of hepatocellular carcinoma. In prospective studies, NAFLD also associates to higher mortality. The risk of death increases with advancing stages of fibrosis (128)(131).

Fatty liver has detrimental effects on lipid and glucose metabolism. Lipolysis from AT leads to increased FFA flow to the liver via splanchnic circulation, leading to ectopic TG accumulation in hepatocytes. Increased liver TG content correlates strongly with systemic IR. However, the causality from intrahepatic TG accumulation to IR remains elusive. Diacylglycerol (DAG) is a TG metabolite, and DAG levels elevate in obesity due to unsuppressed lipolysis. DAG correlates negatively with insulin-mediated suppression of hepatic glucose production in obese humans ($r=-0.609$; $p=0.012$) (132). Thus, DAG accumulation in hepatocytes is a plausible mechanism linking hepatic IR, impaired insulin signalling, and elevated TG content. Yet, the association is not observed in all studies (133). In fasting state, the liver regulates plasma glucose concentration via gluconeogenesis from glycerol, glycogenic amino acids, lactate, and pyruvate, or via glycogenolysis. Liver IR contributes to the failure to suppress glucose production in liver contributing to hyperglycaemia (127)(134). FFAs accelerate the synthesis of TG-rich lipoprotein particles in liver. Very-low density lipoprotein (VLDL) particles are responsible for TG export from liver. However, in NAFLD, is increased VLDL particle production is insufficient to lower intrahepatic TG content (122)(135). Intestinal cholesterol absorption contributes to the

accumulation of free cholesterol (FC) in hepatocytes, even though absorption remains intact in NALFD (136). Simultaneously, the fatty liver induces the activation of cholesterol biosynthesis (136), it increases the de-esterification and cholesterol efflux from hepatocytes is compromised. FC accumulation into hepatocytes aggravates intracellular stress leading to apoptosis and cell death. FC in hepatocytes also activates inflammatory pathways promoting fibrogenesis (130).

Hepatic steatosis and fibrosis are heritable traits correlating more strongly within MZ than DZ twin pairs (137). The well-known genetic loci associating with increased liver fat content are variants of patatin-like phospholipase-domain containing (PNALP3) and transmembrane 6 superfamily member 2 protein (TM6SF2) genes (138). Lipid accumulation to the liver leads to variable clinical outcomes (139)(140). Interestingly, variants of TM6SF2 and PNALP3 genes associate with preserved insulin sensitivity and lower circulating triglyceride levels in NAFLD (139)(141). In contrast – accumulation of hepatic ceramides associate to IR (139). The unique environmental factors when lifestyle, exercise habits or dietary factors, differing between individuals can lead to within-pair discordance in liver fat, even when genetic factors are fully adjusted as documented within weight-discordant MZ twins (37). The differences in the intake of polyunsaturated and saturated fats associate with increased liver fat (65). Furthermore, the intake of fructose stimulates hepatic de-novo lipogenesis (DNL) (142). Weight loss and exercise are first line therapies for NASH, but even more modest weight loss efficiently reduces intrahepatic TG (143)(144).

2.6 Obesity and pro-atherogenic derangements of lipoprotein metabolism

Pancreatic lipase hydrolyses dietary fat TGs into fatty acids (FAs) which are then absorbed by enterocytes in the intestinal lumen. If absorbed FAs are not oxidised to produce energy or used as substrate for cholesterol (C) synthesis in the gastrointestinal tract, they are re-esterified to TGs and incorporated together with apolipoproteins and form soluble chylomicron particles, which enter the blood stream via lymphatic vessels. Lipoprotein particles are mainly synthesised in the liver. The lipoproteins comprise surface apolipoprotein, thin phospholipid layer and core lipids (TGs and cholesterol esters). The higher the protein content, the denser and smaller is the lipoprotein particle. The lipid content is highest in very low-density lipoproteins (VLDL). The lipids gradually decrease, and the proportion of proteins increases from intermediate density lipoproteins (IDL) to low density lipoproteins (LDL). High-density lipoprotein (HDL) particles have the highest protein content of 25–50% (Figure 3). TG-rich chylomicrons and VLDL particles provide the main FA sources for peripheral tissues for their energy demand. Overproduction of TG rich (non-HDL) lipoprotein particles and low HDL-C are characteristic to derangements in lipid metabolism in metabolically unhealthy obesity is (145).

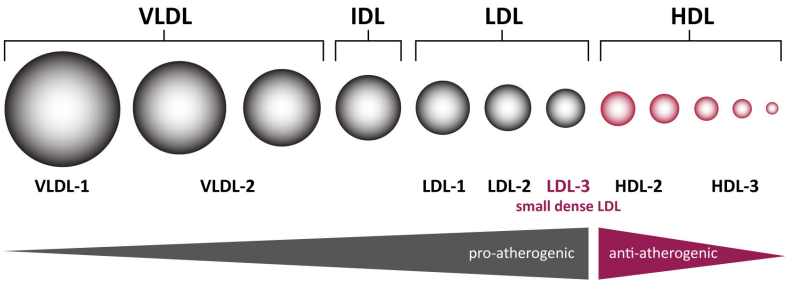

Figure 3 Lipoprotein classes according their size

2.6.1 Hypertriglyceridemia reflects elevated number of large VLDL particles

In obesity, hypertriglyceridaemia mainly reflects elevated TG content in chylomicrons and VLDL particles. Hyperinsulinemia contributes to increased VLDL particle size and number (146)(147). FFA flux from AT due to uncontrolled lipolysis and increased production of apolipoprotein B (ApoB) in fatty liver provide substrates for VLDL assembly. Indeed, in obese subjects with hypertriglyceridaemia, VLDL secretion rate is increased and clearance is impaired, whereas VLDL kinetics is normal in normotriglyceridaemic obese subjects (148). VLDL particles and hypertriglyceridemia has been proposed to be an independent risk factor for CVD beyond LDL-C (149).

Several genetic loci associate with hypertriglyceridaemia and other pro-atherogenic lipid traits (150). As these loci explain an estimated 10-12% of inter-individual variation in TG levels (150), modifiable environmental factors have a significant role contributing to TG levels. A protein-rich diet in relation to carbohydrates or fats has been shown to have to lowering TGs when the overall energy of the diet was adjusted (151). As hypertriglyceridemia can also be reversible after bariatric surgery (152), in spite of the genetic determination, acquired obesity aggravates derangements in lipid metabolism.

2.6.2 Small oxidised LDL-particles promote atherosclerosis

Low-density-lipoprotein (LDL) particles carry the majority of circulating cholesterol (153). Prevalence of hypercholesterolemia is higher in overweight compared with normal weight subjects but it does not increase linearly with BMI (17). Hyperinsulinemia and NAFLD correlate strongly and positively with the LDL-C concentrations and also LDL particle number (146). In metabolically unhealthy obesity, LDL particles undergo qualitative changes that promote atherosclerosis. Increased VLDL TGs are predictive for small LDL size (154). Cholesterol transfer between lipoprotein particles and TG hydrolysis from LDL result in the formation of small and dense LDL particles (154). The small LDL particles are susceptible to oxidation which promotes their atherogeneity (155). Oxidised small LDL (oxLDL) particles accumulate within macrophages, resulting in the formation of foam cells. The lipid-engorged macrophages release pro-inflammatory cytokines contributing to inflammation of the arterial wall, and inhibits nitric oxide release and vasodilation (155). The catabolism of LDL is suppressed. In particular, the clearance of small LDL particles by hepatic LDL receptors is reduced (155). Thus, the formation of small and dense LDL particles strongly associates with pathophysiology of vascular events and increased

cardiovascular risk in obesity (155)(156). Longitudinal studies have shown vascular atherosclerotic lesions manifest decades before the clinical disease in obese subjects (157).

2.6.3 Decreased number and diminished size of HDL particles

High-density lipoprotein particles (HDL) are responsible for reverse cholesterol transport from peripheral vessels to liver for cholesterol secretion to bowel for disposal. HDL associates with anti-inflammatory and anti-atherogenic properties correlating negatively with insulin resistance and risk of CVD (158). HDL particles are heterogeneous and can be divided into subclasses with varying function and size (159). The largest particles have the highest capacity for cholesterol transport. In obesity, HDL-C concentration (17) and HDL particle number are reduced, and particle size diminished (160). The net effect is reduced capacity for reversed cholesterol transport. The prevalence of low HDL-C yields up to 30–40% in obese subjects (17) and is a highly heritable trait (160).

AT distribution (ia and ectopic preponderance) and its function in obesity contributes to proatherogenic dyslipidemia. AT is a major source of free cholesterol and the adipocytes transfer cholesterol to HDL particles (161). Additionally, hyperglycaemia, IR, and abundance of FFAs inhibit ApoA1 synthesis in liver (158). AT contributes to lipidation of apolipoprotein A1 (ApoA1), the main surface protein in HDL. The cholesterol transfer from AT to new HDL particles is reduced in adipose tissue inflammation (161). The plasma cholesteryl ester transfer protein levels are increased in obese subjects making this a plausible mechanism for the reduction of serum HDL-C levels in obesity (162).

The overproduction of large VLDL particles, especially when liver is fatty, initiates a sequence of events that results in the atherogenic lipid profile with low HDL-C (145). Hypertriglyceridemia alters HDL functional properties by impairing anti-inflammatory and vasoactive capacity against oxLDL-induced vasoconstriction (163). Low HDL-C can also be secondary to elevated serum levels of inflammatory cytokine levels without concomitant hypertriglyceridemia (158)(164). In metabolically unhealthy obesity, AT releases inflammatory adipokines such as tumour necrosis factor alpha (TNF-α) that influence both on the loss of anti-inflammatory properties and decreased HDL particle number (164).

> *The genetic factors explain a considerable amount of the variation of the levels and the composition of plasma lipoproteins but the contribution of acquired environmental factors remains important. The data describing the magnitude of environmental contribution of lipoprotein metabolism lacks the genetic control effect. Earlier studies describe the quantitative measures well, in addition to alternations in the cholesterol and lipoprotein particle concentrations in obesity. Whether the composition of lipoprotein particles in different particle classes changes with acquired obesity is previously uncharacterised. Interestingly, some obese individuals remain free from atherogenic lipid derangements. As described above, the fatty liver induces alternations in lipoprotein metabolism. However, not all subjects develop NAFLD. To complement the current data, a twin study is useful to assess the magnitude of the contribution of the environmental factors on lipoprotein metabolism in acquired obesity. Taking account for the clustering of abdominal obesity and dyslipidemia, twin studies can answer questions on the genetic co-variance of atherogenic lipid profile and liver fat.*

2.7 Prothrombotic state in obesity

The formation of a blood clot is a key event in the pathogenesis of arterial and venous thrombotic complications. Blood coagulation is a cascade where activated coagulation factors (F) and platelets together form a fibrin clot. In the clot lysis, plasminogen is converted to active plasmin by tissue plasminogen activator and urokinase, resulting in fibrin degradation products (Figure 4). The clot formation and lysis are dynamic events. In obesity, NAFLD associates with the excess production of coagulation factors in liver (165)(166). Additionally, synthesis of coagulation factors occurs also in AT (167)(168). The low-grade inflammation in AT promotes the release of pro-inflammatory adipokines and cytokines. Subsequently, inflammation fuels the production of coagulation factors promoting the pro-thrombotic state in obesity. The adiposity associates to the defective lysis of fibrin clots.

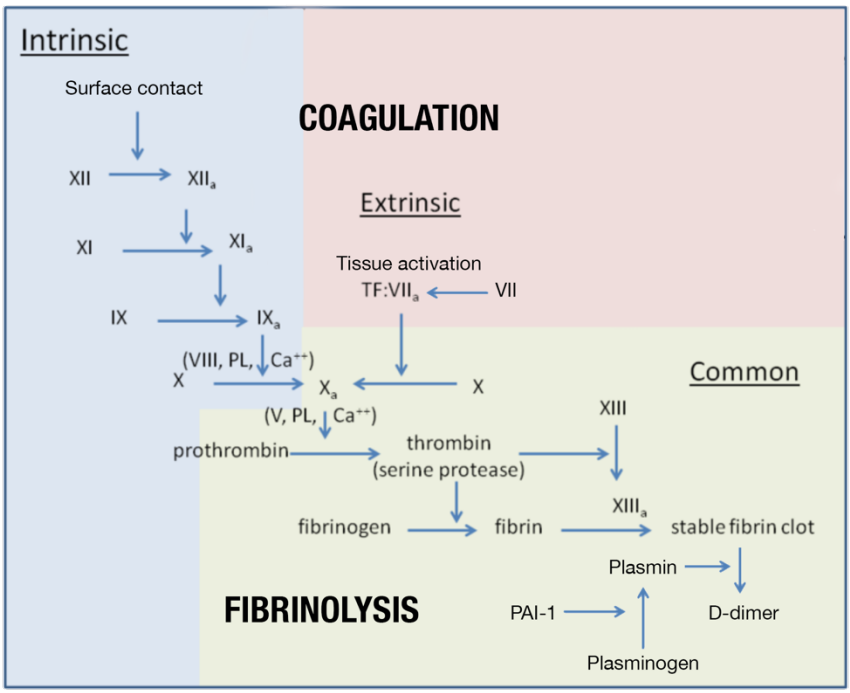

Figure 4 Blood coagulation cascade and fibrinolysis

Figure modified from Pallister CJ, Watson MS (2010). Haematology, Scion Publishing pp 336-47. VII- XIII, blood coagulation factors; $_a$, activated from; PAI-1, plasminogen activator inhibitor-1; TF, tissue factor; PL, platelet membrane phospholipid; Ca^{++}, calcium ion

2.7.1 Platelet hyperaggregation

In formation of thrombus, activated platelets aggregate on fibrin net. Unhealthy obesity associates with platelet hyper-responsiveness, promoting platelet aggregation with fibrin. The underlying molecular mechanisms are complex, including altered platelet size and the enhanced turnover rate, increased biosynthesis of thromboxane, as well as platelet surface adhesion molecules and their ligands. Platelets' resistance to pro-thrombotic signals depend on insulin receptors' sensitivity to insulin (169)(170). The platelet IR is hypothesised to be

the driver behind hyperaggregation and independent from systemic low-grade inflammation (171).

2.7.2 Hypercoagulation

Obesity modifies the activity of the coagulation cascade and alters the levels of several coagulation factors (167). Fibrinogen, as well as von Willebrand factor (vWF), FVII and FVIII levels rise in obesity (168)(172)(173). Fibrinogen is an acute phase protein and elevated fibrinogen levels associate with low-grade inflammation and pro-inflammatory cytokines produced by AT macrophages (174). vWF is mainly synthesised by vascular endothelium (175). It stabilises FVIII re-enforcing its pro-coagulative capacity. Indeed, the levels of these two coagulation factors are typically linked (168). FVII levels correlate positively with hypertriglyceridemia (176), and dietary fat intake has been shown to elevate VII activity postprandially (168)(177). Adipokines leptin, visfatin, and resistin contribute to procoagulative state (167)(178). Leptin and leptin-receptor-deficient mice are protected from arterial thrombosis, the role of leptin in prothrombotic state may be causal (179).

The levels of coagulation factors are heritable, suggesting strong genetic control (180). However, the results from weight loss (181)(178)(182) and dietary intervention studies (167) indicate the pro-coagulative state is modifiable. Upon weight loss, the levels and activity of coagulation factors decrease (182)(183). The levels of coagulation factors associate positively with insulin resistance and low-grade inflammation, suggesting that the genetic regulation of the levels of coagulation factors may be pleiotropic. The function of the same gene may influence both metabolic traits and the coagulation factors (184). The FXIII-A gene is an interesting novel finding and it expresses in the sc AT (185). The FXIII gene polymorphism has been proposed to one of novel candidate genes in the in development of obesity. FXIII-A is a negative regulator of adipogenesis. Located on the surface of preadipocytes, FXIII-A has an inhibitory effect on preadipocyte differentiation (186).

2.7.3 Impaired fibrinolysis

Impaired fibrinolysis characterises metabolically unhealthy obesity. Plasminogen activator inhibitor-1 (PAI-1) inhibits the activity of the tissue plasminogen activator and urokinase, and thereby inhibits the conversion of plasminogen to clot-lysing plasmin. Adipocytes (168) and AT stromal cells, including macrophages, produce PAI-1 (187) and the circulating levels of PAI-1 rise in obesity (188). The levels of PAI-1 correlate strongly with the amount of VAT (187) and therefore PAI-1 is a surrogate marker for ectopic fat deposition. VAT, more than sc or femoral fat, contributes to PAI-1 levels (189)(190). Locally in AT, PAI-1 is involved with adipose tissue differentiation, which prevents angiogenesis and predisposes to hypertrophic adipogenesis, increased adipose tissue stroma cellularity, and faster weight gain (191). In contrast, PAI-1 levels drop during caloric restriction (192). The circulating levels of PAI-1 drop as an immediate response to obesity surgery (193) before a significant drop in body weight. Thus, it is not only the reduction of fat mass explaining such an acute change, but other factors besides adiposity play a role. The genetic polymorphism of PAI-1 has clinical importance as certain gene variants of PAI-1 contribute to development and severity of cardiovascular disease (194).

> *The previous data on hypercoagulable state in obesity does not fully cover the entire coagulation cascade but focuses mainly on fibrinogen, von Willebrand factor,*

FVIII, and fibrinolytic marker PAI-1, while many other factors remain less characterised. The heritability of coagulation factors is high. When validating the results of the obesity-induced alterations in coagulation and fibrinolysis in MZ twin cohorts, results can reveal new associations and pathways to the common genetic basis. The genes regulating the coagulation cascade may be pleiotropic, and the same genes may regulate the levels of several coagulation factors simultaneously. The BMI-discordant MZ twin comparison permits the control of several confounders, including genetic factors.

2.8 Systemic low-grade inflammation in obesity

Systemic low-grade inflammation accompanies the metabolic derangements of MetS, cardiovascular disease and diabetes. Underlying pathophysiologic mechanisms include impairment of insulin signalling and deterioration of glucose uptake in insulin-sensitive tissues (195). Additionally, inflammation also impairs the reverse cholesterol transport pathway. The change in lipoprotein profile favours atherogenesis and cholesterol plaque instability. The levels of pro-inflammatory cytokines such as TNF- α, interleukin-6 (IL-6), and hsCRP elevate in obesity. AT contributes to pro-inflammatory milieu by secreting numerous cytokines (196).

Leptin is a regulatory hormone with multiple roles in the immune system. It activates the immune system, enhances its function, and induces immune cell proliferation (197). In contrast, the role of adiponectin contradicts the anti-inflammatory switch in AT macrophages and suppresses their pro-inflammatory cytokine release (198). TNF- α inhibits adiponectin synthesis (199). Oxidative stress, potentially due to mitochondria dysfunction in obese AT, associates with low-grade inflammation. In dysfunctional AT, the vasculature surrounding hyperplastic adipocytes is underdeveloped and this may lead to diminished blood flow inducing hypoxia (200)(201). Yet, the role of AT hypoxia in human obesity is complex and not fully understood.

2.8.1 Macrophages pro-inflammatory characteristics associate to adipocytes' apoptosis in AT

Infiltration of immune cells is characteristic of obese AT. The number of macrophages correlates with the overall adiposity (202). The number of macrophages increases when maximum lipid storing capacity in AT adipocytes is reached (203). Indeed, the adipocyte hypertrophy promotes macrophage recruitment and the number of macrophages can increase by up to 50% of all AT cells (204). Macrophages cluster together and surround dying adipocytes forming crown-like structures (CLS). The lifespan and the turnover rate of adipocytes in obese adipose tissue is similar to lean state (205). However, the overall adipocyte number is higher in obesity, therefore dying adipocytes are more frequent (205). The role of macrophages is to clear the cell and lipid debris, such as triglycerides phospholipid membrane derivatives (204). The number of macrophages diminishes after weight loss as shown post-operatively in patients that have undergone gastric bypass (206).

Macrophage infiltration does not trigger the systemic immune response in obesity. Macrophage surface markers differ between healthy and unhealthy subjects, and pro-inflammatory markers are more abundant in unhealthy AT (207)(208). Lean AT immune cells are predominantly macrophages polarised to M2 state, so called 'alternatively activated macrophages' (208). The high levels of adiponectin associate with anti-

inflammatory immune response (209). In obesity, low adiponectin levels and adipocyte hypertrophy promote macrophage polarisation to pro-inflammatory M1 state (209)(210). The expression of stress-markers on surfaces of hypertrophic adipocytes may influence the switch in polarisation (195)(209).

In CLS, macrophages are predominantly polarised to M1 state, and secrete pro-inflammatory cytokines attracting more immune cells to the area (210). Inadequate clearance of necrotic cell material is a strong pro-inflammatory signal fuelling AT inflammation. Furthermore, lipid-filled macrophages act as a buffer preventing lipid spill from AT to ectopic tissues (204). In conclusion, the shift in macrophage polarisation is crucial in the development of low-grade inflammation that associates to metabolic complications in obesity (195)(204).

2.8.2 Cytokines and intracellular inflammatory signalling

The cytokines released from adipocytes, AT immune cells, and stroma mediate the insulin signalling in AT (211). TNF-α, IL-1β, and IL-6 impair insulin signalling in AT and correlate with insulin resistance (212)(213). In contrast, IL-10 is a potent insulin sensitiser (214). Hyperglycaemia and free fatty acids induce the production of pro-inflammatory cytokines activating the inflammasome, the intracellular inflammatory signalling pathways (195). Molecular mechanisms between inflammation and insulin sensitivity are complex. The cytokines activate intracellular inflammatory signalling pathways via ligands and receptors on the cytoplasmic membrane (211). Inflammatory signalling pathways interact and inhibit phosphorylation of proteins in the early insulin signalling route. They also contribute to the generation of reactive oxygen species in mitochondria intensifying endoplasmic reticulum stress and activating nuclear transcription of pro-inflammatory and cytokine genes maintaining the inflammatory state (215). Eventually, altered nuclear gene transcript responses locally in AT broaden to systemic pro-inflammatory signals.

Some twin studies suggest genetic factors contribute to variation in the immune response (216)(217). In contrast, the role of lifestyle factors is evident, as weight loss associates with decreased levels of pro-inflammatory cytokines and improved insulin sensitivity (212). Additionally, the immune response is modifiable by diet (211)(218)(219).

2.8.3 The multiple roles of the complement system in AT

The level of the immune response requires constant monitoring. Imbalance in immune system activation may lead to an inappropriate level of inflammation and tissue damage, manifestations of autoimmune diseases, or immune deficiencies. The complement system clears tissue debris and monitors low-grade- inflammation in AT, mainlining the tissue integrity. The complement system consists of membrane-bound or soluble circulating proteins. Hepatocytes synthesise most complement components, but AT adipocytes and stroma also play an important role in their production. In fact, complement factor D, also known as adipsin, is a well-known adipokine (220). Interestingly, the complement components C1q and mannose-binding lectin (MBL) are structural homologues to adiponectin (221).

The complement system is enzymatically activated via three routes, the classical, the alternative, and the lectin pathways (Figure 5). All three pathways combine and lead to the common terminal pathway and eventually to formation of membrane attack complex

(MAC). Characteristic to MAC attack is the rupture of cell membranes, and consequently the cell dies. MAC attack is typical at the sites of bacterial infections. Additionally, the complement proteins prime antigens for opsonisation, and take part in humoral immune defense, chemotaxis, and leucocyte recruitment (222)(223).

Complement system activation has systemic effects also outside AT. Complement component 3 (C3) is the most abundant protein in human sera and its levels elevate in obesity (224). C3 is a biomarker for unhealthy metabolism and together with other complement proteins acts as a biomarker for NAFLD (225)(226). The activation of the complement system correlates positively with the components of the MetS and CVD. Together with complement factor H (CFH) and factor B (CFB), C3 associates positively with BMI, waist circumference, triglycerides, and inflammatory parameters, and negatively with insulin sensitivity and HDL cholesterol (227)(228). Additionally, the elevated levels of C3 and C4b independently predict future type 2 diabetes (229). The complement activation pathways intertwine with those of the blood coagulation cascade and the formation of thrombus. Interactions between the immune system and blood clotting are dynamic. Coagulation factors XIa, Xa, and IXa, and plasmin, act as natural C3 and C5 convertases cleaving C3 and C5 (230). In contrast, the complement components C3, C5, and C5AR1 activate the coagulation cascade and tissue factor (231). These interactions seem clinically relevant – as described earlier, the obesity associates with prothrombotic state.

In AT, instead of MAC activation and cell lysis, the complement system is a key player in the protection of healthy cells from pro-inflammatory immune response from dying and necrotic adipocytes (222)(223). The complement system, as well as adiponectin, clear foreign material by recognising patterns of immune complexes or carbohydrate structures (221). Interestingly, the acylation stimulating protein (ASP, C3a desArg), a biomarker of complement activation, stimulates glucose and free fatty acid uptake in AT enhancing energy storage (232)(233).

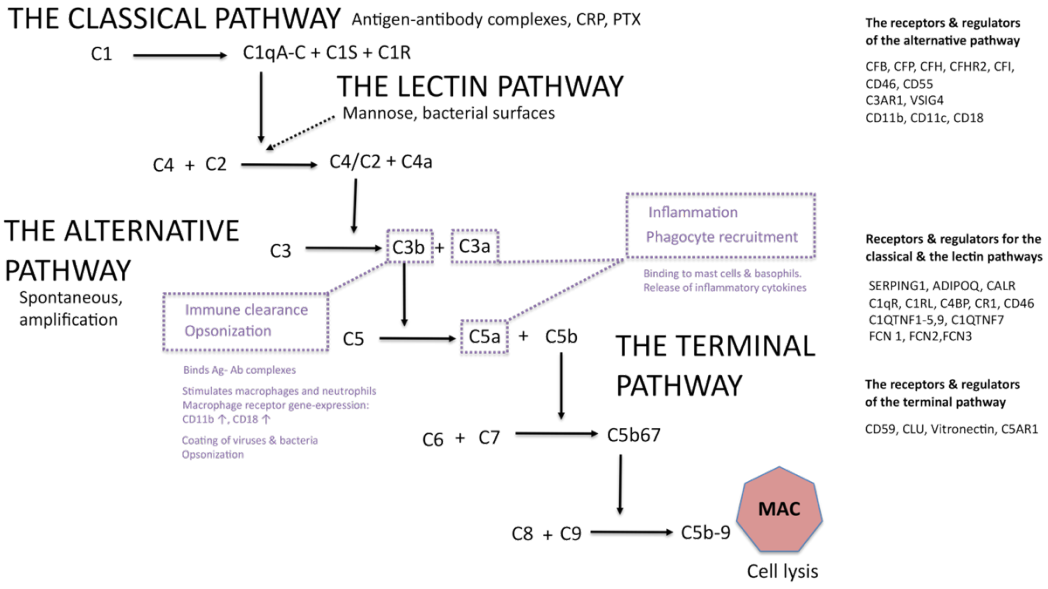

Figure 5 Complement system pathway and its receptors and regulators

Modified from Kaye et al. Front Immunol. 2017; 8: 545. doi:10.3389/fimmu.2017.00545

Low-grade inflammation in obesity predisposes to diabetes and CVD disrupts the glucose and lipid metabolism. The complement system is an important regulator of immune response and its role in AT preceding the manifest clinical disease is not completely understood. It is important to assess how acquired obesity alters the expression of the genes regulating the complement system activation in AT. Since immune cells are abundant in AT stroma, it is intriguing whether hypertrophic adipocytes have independent role in induction of alterations in obesity- induced complement gene-expression transcripts. When comparing the sc AT gene-expression profiles of obesity-discordant MZ twins with well-characterized clinical phenotypes this will give insight on the role of lifestyle in the regulation of innate immune responses.

2.9 The role of the twin studies in the obesity research

2.9.1 Classic twin study design

Twin pregnancies comprise 1.5 % of all pregnancies (234) Finland. Dizygotic (DZ) twins are born when two eggs are fertilized by two different sperms. Therefore, they share 50% of their genes being as similar as any siblings. Monozygotic (MZ) twin pregnancies comprise of 30% of all twin pregnancies (235). MZ twins share 100% of the genes, therefore often are considered "identical", and resemble each other more than DZ pairs. Some phenotypic traits, such as eye colour, are determined by genes. In contrast, some traits are more complex, such as height or body weight, are affected by several genes or interplay of genes and environment. Different alleles, the genes that code the same trait, determine the variance of genetic effect. MZ and DZ twins are often raised together. Therefore, the twin pairs share many environmental factors, such as socio-economic class, upbringing or nutrition. MZ twins' unique environmental factors such as individual habits and lifestyle eventually influence on genetic variance and the penetration of a phenotypic trait.

2.9.2 Discordance within monozygotic twin pairs arise from differences in unique environments

By comparing similarities of MZ and DZ twin pairs, it is possible estimate the heritability of different traits. The heritability estimates elucidate how much of the variance of certain trait is due to genetic effects among individuals. Heritability of BMI is overall high indicating the body size is strongly genetically controlled (29). The heritability estimates of BMI show variation depending on study design and population selection. The influence of the genetic factors is greatest influence during childhood and declines with age (29)(28). High heritability and genetic dominance of a trait does not mean that lifestyle or other environmental factors would not have impact on phenotype. As an example, the polymorphism in FTO-gene is known to increase body weight and the risk of obesity. However, with successful lifestyle intervention, the carriers of risk allele were able to lose weight (236).

The heritability is typically higher when little variation occurs in environmental factors (237). In Finnish twin cohort, BMI heritability estimates ranged from 0.58 to 0.69 among 11-17 years old (238). The effect of common environmental effects weakened with the age as the effects of unique lifestyles became more prominent (238). Similar observation was noted when analysing the growth patterns of adult identical but BMI-discordant twins

(239). Even if the co-twins were discordant at birth, then the growth appeared similar during childhood. Divergence in growth started re-emerging at the age of 18 leading to significant discordance (within –pair difference in BMI > 3 kg/m^2) in adulthood (239).

Discordance in birth weight is common among twin pregnancies (240) and this suggests twin's unique intra-uterine condition contribute to the expression of genes. The differences within co-twins may emerge as early as during embryogenesis or during intra-uterine growth due to unequal nutrient distribution or placental vasculature (241). In rare cases de novo genetic mosaicism, chromosomal re-arrangements and mutations have led to discordance of the phenotype (241). However, the differences within adult MZ twin pairs usually arise from their own non-shared habits and environmental factors such as smoking, physical exercise or diet.

Differences of the epigenome, the methylation of non-coding DNA and histone acetylation in chromatin, mediate the relationship between genotype and unique environmental factors. The epigenetic differences have impact on the expression of the coding genes (242). Phenotypic differences arising from epigenetic changes seem to augment over the lifespan and lead to heritable differences in gene transcripts within ageing MZ twin pairs (243). Yet, the causality of epigenetic mechanisms as a driver on outcome of complex metabolic traits remains unclear. The discordance in methylation profiles is not established as concluded in obesity-discordant twin study by Souren (244). Interestingly, DNA methylation profile in BMI-discordant twin pairs was similar in healthy obesity without metabolic complications, but differed when obesity associated with metabolic disturbances (245). The evidence from environmental contribution to the outcome of a disease is highly informative for lifestyle intervention and primary prevention of metabolic complications in obesity.

Several studies have addresses the questions whether lifestyle patterns could be genetic. A twin studies showed the genetic variation explain 40% of unhealthy eating patterns as well as contributed to the initiation of smoking (246). Additionally, the genetic variance may explain the intensity of smoking and the amount of cigarettes smoked (246). Data collected from questionnaires on the food-frequency and participation on competitive sports imply the eating and exercise habits are genetic controlled (247).

2.9.3 Obesity-discordance within MZ twin pairs is rare

In MZ twin studies, it is possible to analyse gene-environment contributions by twin-twin comparison of observed phenotypic differences. The comparison MZ co-twins allows the analyses of discordance of a disease or trait in unique case-control setting. Insights on the metabolic consequences of acquired obesity have gathered from carefully characterized sample of population based cohort of BMI-discordant twins from Finland (36). Similar twin cohorts have also been described elsewhere (248)(249). As discordance within MZ twins is rare, the explanation arises from a longitudinal follow-up study showing BMI-discordance in MZ twins is only temporary: only 2,4% of pairs remained discordant over in average 6.4yrs follow-up (248).

Differently expressing genes within MZ co-twins offer a powerful tool to detect possible genetic causality. After detection of a suggestive genetic contribution on a trait, it is possible to continue research to genotyping specific loci on chromosomes and further search for candidate genes. Clustering of the metabolic traits, such as hypertriglyceridemia, insulin resistance and hypertension, is characteristic to obesity. Clustering may imply to

pleiotropic action of the same genes on these traits. In multivariate model exploring aetiology of the coherence of the components of MetS in middle-aged men only adiposity was genetically and environmentally related to all of the metabolic traits (250). This result suggests candidate gene studies on metabolic dysfunction should be aimed towards the genes regulating AT distribution and function.

It is evident that the phenotypes (insulin resistance and metabolic syndrome) are influenced by lifestyle and environmental factors which need to be considered when exploring the underlying genetic architecture of these traits. The classic twin study design however, does not allow the researcher to consider the effects of both shared environment and gene-environment interaction simultaneously. More complex twin analytical models such as Cholesky (251)(252) are used decomposing genetic and environmental correlations between two or more traits.

The prevalence of the obesity-related metabolic diseases has rapidly increased in past decades, yet, the human genome has not changed with the same timeframe. The eating habits may have earlier been considered merely due to unique lifestyle and individual preferences. Now we know that the same genes not actually only regulate the body size but also appear to regulating appetite and satiety. The environmental factors undoubtedly contribute to the function of the genes regulating metabolic pathways and cannot be disregarded. Therefore, this thesis is aimed to complement current evidence and provide more insight on to the gene-environment interaction.

3. AIMS

The objective of this thesis was to explore, in a unique setting of young adult obesity-discordant MZ-twins with identical genes, phenotypic (environmentally determined) changes brought by acquired obesity in blood coagulation, serum lipids/lipoproteins, and complement system. Specifically, in this thesis the following key points were investigated:

I: Changes in blood coagulation factors, measures of fibrinolysis, and the susceptibility to thrombosis in acquired obesity

II: Early changes in lipid metabolism, serum lipoprotein particle quantity, and quality in acquired obesity

III: The magnitude of environmental and genetic contributions to the association between the abdominal obesity and serum metabolites assessed by NMR

IV: Changes in the circulating levels of complement-system proteins and complement gene expression in subcutaneous adipose tissue and isolated adipocytes gene transcripts in acquired obesity

4. SUBJECTS AND METHODS

4.1 Participants

The Finnish Population Registration Centre was the provider of the data of all Finnish twins from five consecutive birth cohorts (253). The twins for obesity-studies were recruited from two population-based longitudinal studies, FinnTwin16 (FT16) and FinnTwin12 (FT12). The twins were invited for interview and to fill questionnaires about their behavioural and health habits. In FT16, twins born 1975–1979 (n=5601 at baseline), were followed up by questionnaires at 16, 17, 18.5, and 22–27 years of age (response rates 83–97%). For the FT12 cohort (n=5184 at baseline), twins born 1983–1987 were sent questionnaires at 11–12, 14, and 17.5 years (response rates 74–92%). Monozygosity was confirmed by genotyping 10 informative genetic markers (254) and later by genome-wide association study (GWAS) platform.

Figure 6 presents the descriptive flow chart of twin cohorts. The TwinFat sample was enrolled from the FT12 and FT16 cohorts based on the reported BMIs in the fourth wave of the data collection. Twins were selected aiming to cover a full BMI range, of both normal-weight and obese subjects, and a full range of within-pair differences in BMI. In addition, 100 twin individuals were chosen randomly with respect to their BMIs, and they were included in the quantitative gene analyses. The TwinFat subsample comprised 286 subjects (136 MZ subjects; 150 same sex DZ subjects; age range: 22.8-36.2; 52.9% female). Altogether 33 MZ twin pairs were discordant for BMI (ΔBMI $\geq$3 kg/m^2; age range: 22.8-36.1; 64% female). The BMI-discordance in MZ twins is extremely rare, and these pairs represent the top 5% of the most discordant pairs of FT12 and FT16 birth cohorts.

Quantitative genetic analyses (Study III) included a larger population of FT12 and FT16 subjects (n=1368; FT12: n=725, FT16: n=543 and TwinFat: n=100; MZ: 531, DZ: n= 837; age range: 21.0-31.5; 52.5% female). The intensive cohort from TwinFat subsample comprised 81 MZ twin individuals (aged 22–36, 54% women). Within the intensive cohort, 26 full pairs discordant for BMI (ΔBMI > 3kg/m^2) were carefully examined 2002-2011. A total of 14 full pairs concordant for BMI (ΔBMI <3kg/m^2) were randomly selected for their controls. Over the year, all pairs had not remained BMI-discordant longitudinally. The data from repeated clinical examinations were analysed as concordant control pairs.

All twin pairs were Caucasian and normotensive, none were pregnant or lactating, and oral contraceptive use was documented. One obese co-twin had T2DM and used metformin and insulin, and one twin had inactive ulcerative colitis and used mesalazine and azathioprine. These individuals came to clinical examination only after 2008, therefore they are not included in study I as the data for analyses was collected earlier. All twins were medically examined affirming their physical and psychiatric health status. The twins' weight had been stable for at least 3 months prior to the examination. The study protocol was designed and performed according to the principles of the Helsinki Declaration and was approved by the Ethical Committee of the Helsinki University Central Hospital.

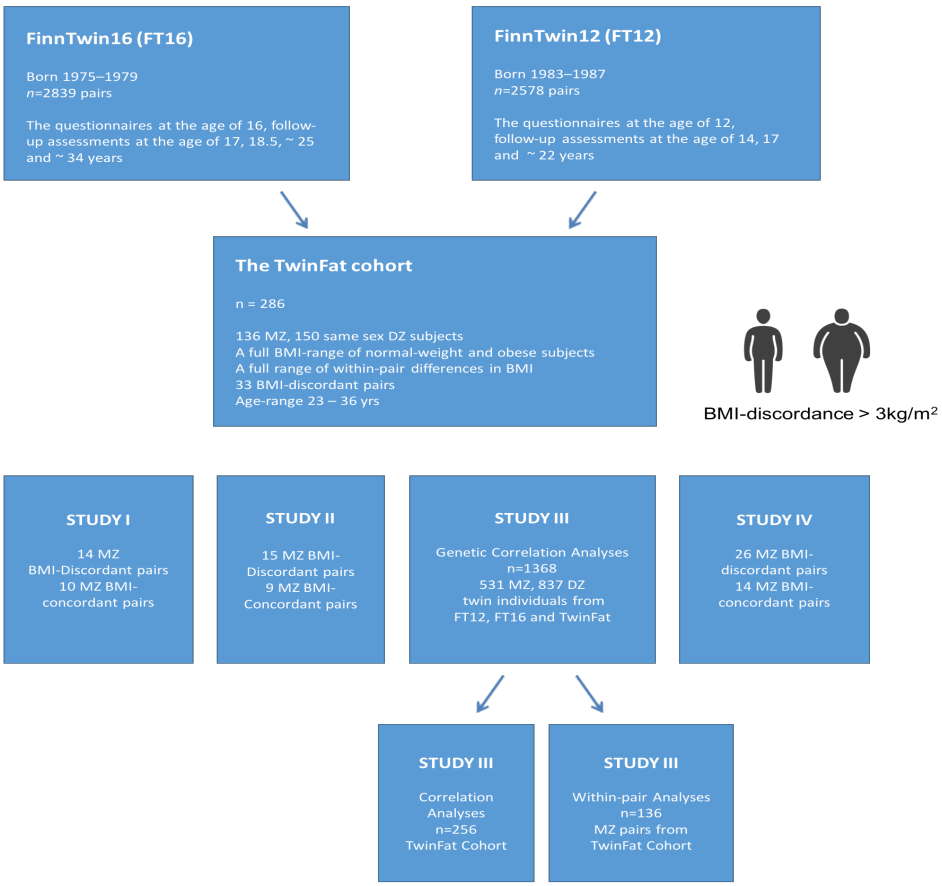

Figure 6 The flow chart of twin cohorts

4.2 Body composition measures and clinical chemistry (I-IV)

A summary of the available clinical and anthropometric measures in Studies I-IV is available in Appendix Table 1. Briefly, weight and height were measured after a 12-hour overnight fast in order to calculate BMI (body mass (kg)/height(m)^2). Body composition was measured using whole-body dual x-ray absorptiometry (DXA) scans (Lunar Prodigy, Madison, WI, software version 8.8). A standardised procedure at least 4 hours after a light meal with empty bladder was used to avoid differences in the hydration status. Whole body fat percentage was calculated as 100x fat mass/(fat mass + lean mass + bone mineral content). The total body and android and gynoid fat mass and fat percentage were determined from a total body scan as described by Wiklund et al (255).

MRI measurements were performed on a clinical 1.5 Tesla imager (Avanto, Siemens, Germany, Erlangen). To allow measurement of abdominal fat distribution, a T1-weighted axial image stack of 16 slices with a thickness of 10 mm and gap of 0 mm was centred at L4/L5 intervertebral disk. Selective fat excitation was used to obtain Images with a standard body coil. MR images were analysed using SliceOmatic v4.3 segmentation software (Tomovision, Montreal, Canada). The areas of sc and ia fat tissue were measured for each slice using a region-growing routine. The results were expressed as total volumes of sc fat and ia fat.

For liver fat analyses, point resolved spectroscopy (PRESS) localisation technique with TR/TE of 3000/30 ms and 16 acquisitions was used to obtain non-suppressed liver spectra.

Orthogonal three plane images were used for localisation of the cubic 8 -27 cm^3 voxel of interest within the right lobe of the liver avoiding signal contamination from vascular structures, gallbladder and adipose tissue. The MRS data was collected using a flex surface coil in combination with spine coils. The liver spectra were analysed with jMRUI v3.0 software (256) by using the AMARES algorithm (257). Areas of water signal at 4.7 parts per million (ppm) and methylene signal from intracellular triglycerides at 1.3 ppm were determined using a line-fitting procedure. Spectroscopic intracellular triglyceride content was expressed as methylene/(water+methylene) signal area x 100 and values were further converted to mass fractions as earlier described by Kotronen et al. (258).

Blood samples were collected from antecubital veins at 8:00-8:30 am after fasting 10-12 hours overnight. Plasma and serum were separated by centrifugation for 15 min at 2000 g at 4 ºC. The separated plasma and serum were frozen in aliquots at -70 ºC until assayed. In studies I-IV, the serum insulin was analysed with time-resolved immunofluorometric assay (Perkin Elmer, Waltham, MA, USA) and high sensitivity C-reactive protein (hsCRP) (Cobas CRP (Latex) HS, Roche Diagnostics). Other laboratory analyses from plasma/serum are listed in Appendix Table 2.

4.3 Euglycaemic hyperinsulinaemic clamp (Study I)

In Study I, insulin sensitivity was determined by the euglycaemic hyperinsulinaemic clamp technique (259). Two 18-gauge catheters (Venflon; Viggo-Spectramed, Helsingborg, Sweden) were inserted: one to an antecubital vein to be used for infusion of insulin and glucose; a second inserted retrograde in a warmed hand vein to obtain arterialised venous blood for measurement of plasma glucose concentrations (every 5 min) and serum free insulin concentration (every 30 min). The rate of the continuous regular human insulin (Insulin Actrapid; Novo Nordisk, Denmark) infusion was 40 $mU/m^2 \cdot min$ (1 mU/kg·min) for 120 min. Normoglycaemia was maintained by adjusting the rate of a 20% glucose infusion based on plasma glucose measurements from arterialised venous blood. Insulin sensitivity was determined from the glucose infusion rate needed to maintain normoglycaemia after correction for changes in the glucose pool size (259). The M-value was expressed as mg glucose/kg fat-free mass per minute and considers that glucose uptake occurs mainly to muscle. Because hepatic glucose production is maximally suppressed in non-diabetic subjects already at an insulin concentration achieved during infusion of insulin at a rate of 0.5 mU/kg·min (260), the M-value reflects the glucose uptake. For study III, HOMA-IR was calculated as fasting plasma glucose*serum insulin/22.5 (261).

4.4. Serum metabolites (Study III)

All serum samples were analysed using the same high-throughput NMR metabolomics platform. Altogether 56 metabolites were selected to provide quantitative information on lipoprotein subclass and particle concentrations, and serum FAs, including omega-3 and omega-6 FAs and low molecular-weight metabolites such as nine amino acids, 3-hydroxybutyrate, and α1-glycoprotein. Altogether 56 metabolites were selected and analysed in the current study. The sample preparation and NMR spectroscopy methods have been described in detail elsewhere (262)(263).

4.5 Lifestyle questionnaires, diet, smoking, and physical activity (Study II)

Energy and macronutrient intakes were assessed from three-day food records and analysed by Diet32, established on a national Finnish database for food composition (264). Habitual alcohol intake, smoking, and physical activity habits were assessed using questionnaires. The non-smokers comprised never smokers and former smokers, and the current smokers comprised those who were daily or occasional smokers. Baecke physical activity scores comprise an index for physical activity in total (total index) and separate indices for physical activity at work (work index), sports activities during leisure time (sport index), and physical activity during leisure time excluding sports (leisure time index (265).

4.6 Adipose tissue biopsies (Study IV)

Adipose tissue (AT) biopsies were obtained from periumbilical sc fat using a surgical technique. RNA was prepared from frozen fat. The diameter of fat cells was measured under a light microscope from fresh biopsies treated with collagenase. From biopsy samples, the transcriptomics experiments were performed with Affymetrix U133 Plus 2.0 chips. Raw gene expression data were preprocessed with the GeneChip robust multiarray averaging (GCRMA) algorithm using BioConductor in R (266) and by using Brainarray custom cdf for probe annotations (267), and were validated with qPCR as in the study by Naukkarinen et al (37).

4.7 Statistical methods

Statistical analyses were performed using the Stata statistical software (releases 9.0-11.0; Stata Corporation, College Station, Texas). Survey data procedures were used to correct for clustered sampling of co-twins within pairs. Log- and rank transformations were used to normalise the distribution of non-normally distributed data. Wald and Mann-Whitney tests for independent samples (=t-tests adapted for clustered twin data) were used to compare groups between samples. Pearson correlations, with correction for clustering, were calculated to determine the relationships between clinical and biochemical and gene-expression parameters in individual twins (I-IV). The co-twins were compared by Wilcoxon's signed ranks test (I-IV). Male and female pairs were combined because MZ co-twins are matched for gender by definition. Spearman correlations of within-pair differences in body composition, inflammation and metabolic measures were used to test the effects of the extent of adiposity discordance (I-IV). Because MZ twins are identical at the sequence level and share all their segregating genes, the associations within MZ pairs are fully adjusted for genetic effects. Twin similarity was assessed using intra-class correlations (ICC)(I-II). Multiple regression analyses were performed to detect independent predictors of observed differences within twin pairs.

4.7.1 Quantitative genetic model fitting (III)

The classical twin study design considers two types of genetic and two types of environmental influences. The additive genetic influences (A) result from the cumulative effects of genes. The dominant genetic effects (D) result from interactions between alleles at the same locus (dominance) or different loci (epistasis). The common environmental influences (C) make the twin pair similar for the trait, and the unique environmental effects (E) include all environmental factors and experiences that make the twin pair dissimilar. The latter (E) also includes measurement error. MZ twin pairs correlate 1 for both A and

D, whereas DZ pairs correlate 1/2 and 1/4 for these components, respectively. Both MZ and DZ pairs correlate 1 for C. E is uncorrelated for both types of twins by definition. Different combinations of these components (e.g. ACE, ADE, AE and CE) can be hypothesised to account for the pattern of variation and covariation in twin data. Dividing each of these components by the total variance yields the different standardised components of variance, such as heritability, which is defined as the proportion of the total variance attributable to genetic variation.

In Study III, the quantitative genetic model fitting was performed to assess the contributions of genetic and environmental factors to the co-variation of abdominal obesity (expressed her as WC) with circulating serum metabolites. In brief, twin methodology makes use of the fact that MZ twins share 100% of their segregating genes, whereas DZ twins share an average of 50% by descent. Given the significant associations between abdominal obesity and most serum metabolites, a bivariate Cholesky decomposition model **(**Figure 7) was used to examine the genetic and environmental contributions to the covariance between WC and 56 serum metabolites. The first factor (A1, E1) contributes to both variables (i.e. WC and the metabolite), and the second factor (A2, E2) reflects influences specific to the second variable, thus the metabolite (251). Whether the best-fitting bivariate model was ACE, ADE, AE or CE for WC and for the metabolites was determined by considering both goodness-of-fit and parsimony in explaining the data. Mx, a structural equation modelling program, was used to perform the model-fitting analyses (252).

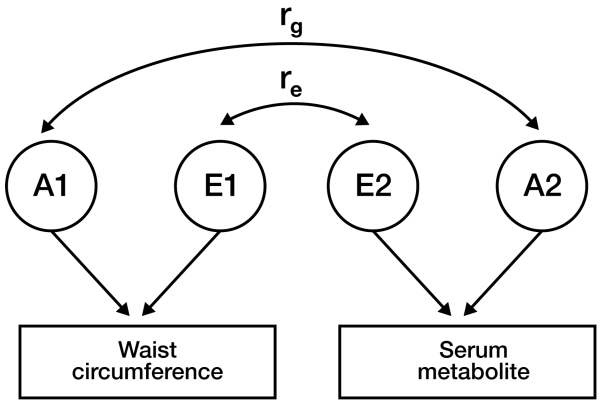

Figure 7 Schematic presentation of Cholesky decomposition model to assess the genetic and environmental contributions to the covariance between WC and metabolites profile

r_g, genetic correlation, r_e environmental correlation

5. RESULTS

The focus of the analyses in studies I–IV was to characterise and compare phenotypes of BMI-discordant MZ twin pairs. Their clinical characteristics are summarised in Table 1.

5.1 High overall within-pair similarity of BMI-concordant MZ twin pairs (Studies I–II)

In studies I–II, within pair intra-class correlations (ICC) were calculated in order to quantify the degree of the resemblance of the MZ co-twins with in their phenotypic measures of coagulation factors and lipid parameters. Figure 8 demonstrates ICCs of selected biomarkers. The coagulation factors and lipoprotein particles appeared to be under strong familial influence in both concordant and discordant MZ twin pairs (r=0.48–0.97, p<0.05). However, with BMI-discordance, the magnitude of the familial influence was weaker. The correlation estimates (r) for total cholesterol were more significant within concordant (r=0.88, p<0.001) than discordant pairs (r=0.58, p<0.01). For Factor IX (FIX) the ICC within discordant pairs was non-significant (r= 0.34, p=ns) whereas within the concordant pairs the ICC was highly significant (r=0.86, p<0.001). The ICCs for all measured lipoprotein parameters of the concordant pairs were high and statistically significant (0.70–0.85, p<0.01). However, in BMI-discordant pairs, ICCs for VLDL, ApoB, VLDL-C, LDL-C, HDL2a%, and total TG concentration were low (r=0.11–0.35, p=ns) and no significant familial resemblance occurred.

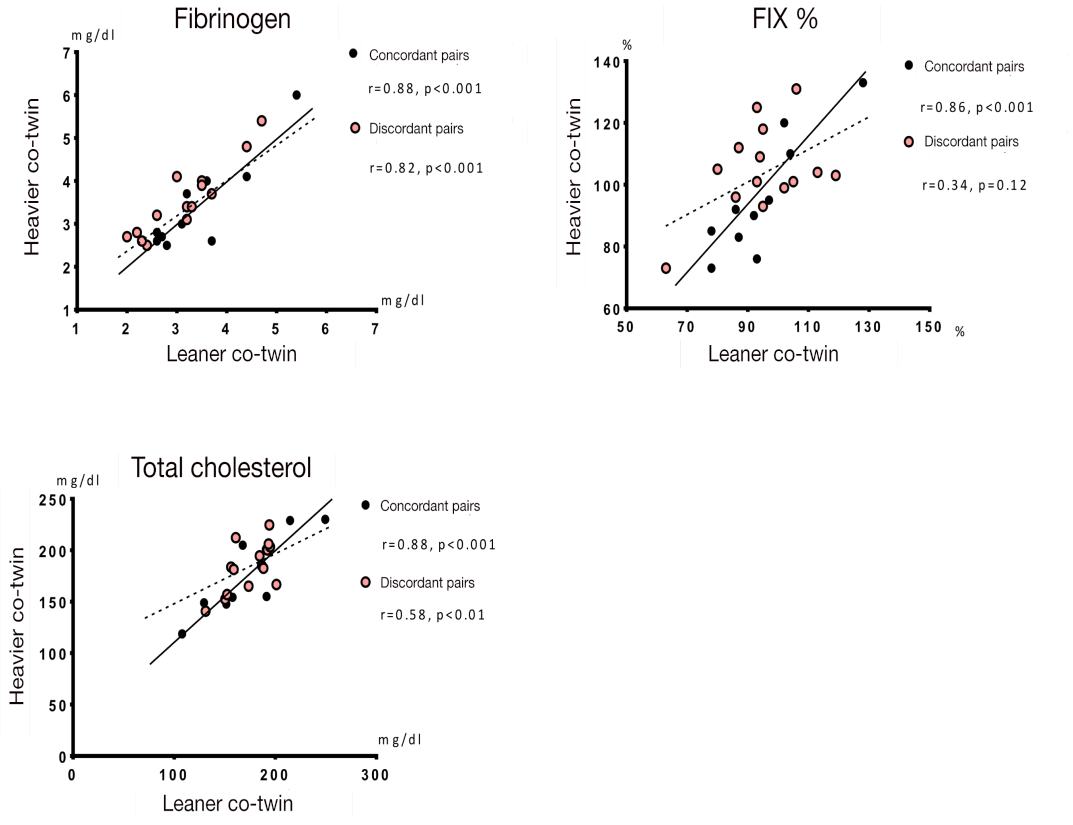

Figure 8 Intraclass correlations within MZ twin pairs either concordant or discordant on obesity

r, correlation co-efficient, FIX, coagulation factor IX

Table 1 The Clinical characteristics of subjects in studies I- IV

	Study I			Study II			Study III				Study IV		
	BMI-discordant pairs n=14		Concordant twin individuals n=18	BMI-discordant pairs n=14		Concordant twin individuals n=9	FinnTwin cohort n=1356 individuals	Twin Fat n=286 twin individuals	BMI-discordant MZ pairs n=33 pairs		BMI-discordant pairs n=26 pairs		Concordant twin individuals n=28
	Leaner	Heavier		Leaner	Heavier				Leaner	Heavier	Leaner	Heavier	
BMI kg/m2	25.3 ± 0.5	30.5 ± 0.5	26.8 ± 1.6	24.8 ± 1.0	30.3 ± 1.0	28.4 ± 0.8	23.6 ± 0.1	25.4 ± 0.3	25.3 ± 0.9	31.3 ± 1.0	25.3 ± 0.9	31.3 ± 1.0	26.9 ± 0.7
Fat %	29.7 ± 2.4	40.2 ± 2.1	26.4 ± 2.7	32.2 ± 2.4	40.6 ± 2.0	32.3 ± 2.0	n/a	29.0 ± 0.8	31.7 ± 2.0	40.9 ± 1.5	32.25 ± 1.81	41.14 ± 1.32	28.9(24.1-35.3)
Waist cm	88.9(85.7 - 93.3)	103.1(98.6-105.6)	83.6(78.3 - 98.4)	83.4 ± 3.1	97.4 ± 3.1	90.5 ± 2.1	80.9 ±0.4	87.3 ± 0.9	83.8 ± 2.3	99.6 ± 2.5	80.9 (74.3-94.3)	96.7 (89-110)	88.4(79.7-94.2)
Sc fat dm3	2.7(2.4-4.0)	4.8(4.4-5.8)	2.7 (1.4-4.2)	3.4 ± 0.4	5.4 ± 0.1	3.3(2.6–5.1)	n/a	n/a	3.2(2.3-4.8)	5.7(4.3-7.8)	3.2(2.4-4.9)	5.7 (4.3-8.0)	2.8(2.4-4.0)
Ia fat dm3	0.5(0.4-0.7)	1.0 (0.8-1.2)	0.6(0.4-1.1)	0.6 (0.3-0.8)	1.1 (0.7-1.5)	1.1(0.6- 1.5)	n/a	n/a	0.6(0.3-0.9)	1.3(0.8-2.2)	0.6 (0.3- 0.8)	1.2(0.7- 2.2)	1.0(0.4-1.5)
Liver fat %	1.25(1-2.5)	3.6(2-11)	1.0(1.0-8.0)	0.6 (0.4-1.1)	3.8 (0.6-6.1)	1.2(0.5- 1.7)	n/a	n/a	0.6(0.4-1.0)	3.4(0.6-7.3)	0.6(0.4-1.0)	2.7(0.6-7.3)	1.0(0.5- 2.0)
Glucose mmol/L	5.1 ± 0.1	5.3 ± 0.2	5.3 ± 0.1	5.1 ± 0.1	5.1 ± 0.1	5.4 ± 0.1	n/a	5.1 ± 0.0	5.1±0.1	5.2±0.1	5.2 ± 0.1	5.3 ± 0.1	5.4 ± 0.1
Insulin mU/L	5 (3-8)	9 (6-11)	6(5-9.5)	4.8 (3.3-6.8)	8.2(5.2-10.3)	5.2(3-8.3)	n/a	6.2 ± 0.3	4.6(3.2-6.5)	7.7(5-10.5)	4.5(3.2- 6.7)	7.5 (4.9- 9.9)	5.2(3.0-6.9)
M-value*	9.2 ± 0.9	6.3 ± 0.6	7.4 ± 0.7	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
HOMA IR	n/a	n/a	n/a	n/a	n/a	n/a	n/a	1.4 ± 0.1	1.0 (0.7-1.5)	1.8(1.0-2.5)	1.0 (0.7-1.5)	1.8(1.0- 2.4)	1.3(0.6-1.7)
hsCRP mg/L	0.9 (0.2-1.1)	2.0 (0.9-3.4)	0.48(0.2-2.0)	1.05 (0.5- 4.1)	1.34 (0.6- 7.7)	0.9(0.5 -1.8)	n/a	1.7 ± 0.2	1.0(0.3-3.0)	1.3(0.6-5.9)	1.0(0.45- 3.93)	1.6 (0.8- 6.3)	0.8(0.3- 1.5)
Trigly mmol/L	1.0 (0.7-1.2)	1.4 (0.9-1.6)	1 (0.75 - 1.7)	1.0 (0.8 - 1.2)	1.1(0.9-1.3)	1.0(0.7- 1.2)	n/a	0.9(0.7- 1.3)	0.8(0.6 -1.1)	1.1(0.8-1.4)	0.9(0.6-1.2)	1.1(0.8-1.4)	0.7(0.6- 1.1)
Chol mmol/L	n/a	n/a	n/a	4.3 ± 0.1	4.6 ± 0.2	4.4 ± 0.2	n/a	4.6 ± 0.1	4.4 ± 0.1	4.6 ± 0.2	4.4 ± 0.1	4.7 ± 0.2	4.5 ± 0.2

* mg glucose/kg fat free mass per minute

5.2 Acquired obesity associates with hypercoagulable state (Study I)

The objective in the study I was to quantify the magnitude of changes in blood coagulation factors and the markers of fibrinolysis in acquired obesity. Despite the strong familial control of the overall activity of the markers of coagulation and fibrinolysis, several differences emerged within BMI-discordant co-twins. The obese co-twins of BMI-discordant pairs had higher levels of fibrinogen, and the activities of factors IX, XI, and XII, as well as PAI-1, were increased (Figure 9). The differences were significant, even the effect of potential confounders, gender, smoking, and the use of oral contraceptives, were adjusted. There were no differences in markers of coagulation, PAI-1, or D-dimer between co-twins of BMI- concordant pairs. Thus, obesity corresponds with the pro-coagulative and hypofibrinolytic state.

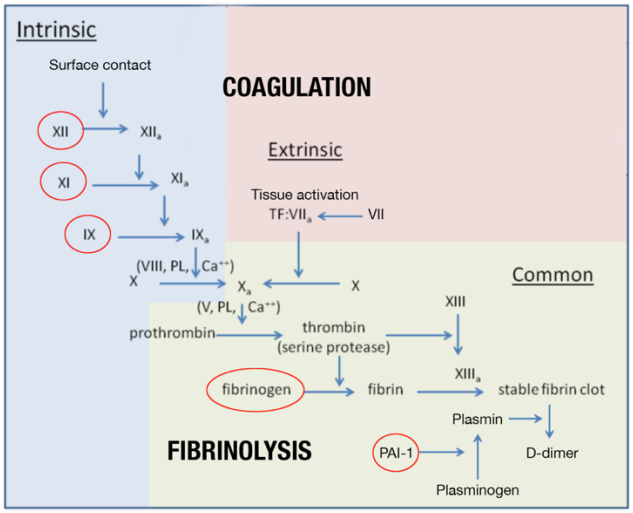

Figure 9 Increased activity of markers of blood coagulation and fibrinolysis in acquired obesity

Figure modified from Pallister CJ, Watson MS (2010). Haematology, Scion Publishing pp 336-47
VII- XIII, blood coagulation factors; $_a$, activated from; PAI-1, plasminogen activator inhibitor-1, TF, tissue factor; PL platelet membrane phospholipid; Ca^{++}, calcium ion

In univariate analyses of twin individuals, the body fat distribution, the amount of sc and ia fat and liver fat, as well as inflammation (hsCRP) associated positively with hypercoagulable state ($r=0.36–0.73$, $p<0.05$ for all). In contrast, the correlations between FIX% and FXI% and insulin sensitivity (M-value) were negative ($r=-0.40$ and -0.54 respectively, $p<0.01$ for both).

To control the genetic effects behind the differences observed within MZ twin pairs, the correlation analyses were repeated and Spearman correlation analyses were performed between the intra-pair differences (Δ) in the anthropometric and metabolic parameters (Δsc fat, Δia fat, Δliver fat, Δinsulin, ΔM-value and ΔhsCRP) and the Δmarkers of the coagulation and the fibrinolysis. ΔFibrinogen levels and ΔPAI-1 activity correlated positively and significantly ($r=0.42–0.70$ and $r=0.52–0.58$ respectively, $p<0.05$) with Δadiposity markers, Δinsulin, and ΔhsCRP. Therefore, the acquired adiposity contributes to procoagulative and hypofibrinolytic state in obesity after genetic background was adjusted for.

5.3 Atherogenic lipoprotein profile in acquired obesity (Studies II–III)

In study II, the quantity and the quality of the fasting serum lipoprotein profile was assessed by ultracentrifugation, gradient gel-electrophoresis, and colorimetric enzymatic methods. In study III, the analyses of lipoprotein subfractions were replicated with NMR. The aim was to determine how excess body fat and its distribution in obesity reflects the proatherogenicity in lipoprotein measures. The correlations between overall obesity, body fat distribution and the measures of insulin resistance (fasting insulin, glucose and HOMA-IR) and lipoprotein particle profile was analysed. Additionally, the data from 3-day food diaries and questionnaires on physical activity (Study II) was available after complementing the assessment of the role of the lifestyle factors.

5.3.1 Increase in proatherogenic lipoprotein particles (Studies II–III)

The heavier co-twins of BMI-discordant pairs had more atherogenic lipoprotein profile compared with their leaner co-twins. They had significantly higher concentrations of ApoB as well as total, IDL- and LDL-C. The total IDL and LDL particle mass was higher in obese subjects. Obesity induced alterations in HDL-subfractions. The concentrations of HDL-C and HDL2-C and proportion of HDL2b were lower and HDL mean particle size was smaller (Study II). Obese co-twins had a higher proportion of small HDL3c particles in comparison to leaner co-twins.

In the heavier co-twins of BMI-discordant pairs, the corresponding pro-atherogenic lipoprotein subfraction profile emerged in NMR (Study III). The heavier co-twins had higher concentrations of VLDL and LDL particles, therefore more ApoB, triglycerides, and cholesterol than their leaner counterparts. The concentrations of HDL particles and HDL-C were lower and HDL diameter was smaller. HDL-to-LDL-ration was lower, and the ApoB-to-ApoA1 was higher in the heavier co-twins.

NMR measurements allowed the estimation of fatty acid profiles. The ratio of poly-unsaturated fatty acid (PUFA) to total FAs was lower in the obese than lean co-twins. All lipid and lipoprotein measures were similar within BMI-concordant co-twins, independent of the method being used in the analyses.

5.3.2 Unaltered lipoprotein particle composition (Study II)

Figure 10 describes the composition of lipoprotein particles in the heavier co-twins of the BMI-discordant pairs. The particle composition of the leaner co-twin resembles the one seen in heavier co-twins (data is not shown). Only subtle changes in the particle composition were seen in obesity: percentage of IDL free cholesterol (7.7% vs. 7.0%, p=0.05) and IDL cholesterol esters (10.6% vs. 9.0%, p=0.05) were elevated. Additionally, HDL2 particles were enriched with TGs in the heavier co-twins (6.1% vs. 5.1%, p=0.02).

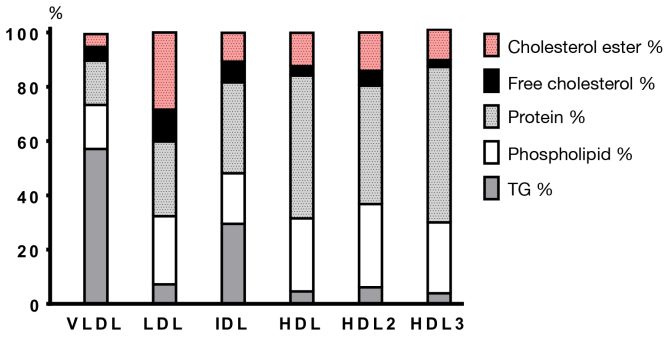

Figure 10 Lipoprotein particle compositions in the heavier co-twins of the BMI-discordant pairs

Taken together, of the alterations in the lipoprotein measures in studies II–III, the lipoprotein particle composition does not change but the number of non-HDL lipoprotein particles increases, and the capacity of HDL particles to carry cholesterol is compromised.

5.3.3 Abdominal body fat distribution associates to atherogenic lipoprotein profile (Studies II–III)

In Spearman correlation analyses within MZ twin pairs the Δatherogenic lipid and lipoprotein parameters correlated significantly ΔBMI (Figure 11). Similar correlations were observed with other Δmeasures of obesity: the Δamount of sc, ia, and liver fat after adjustment of confounders (gender, smoking status).

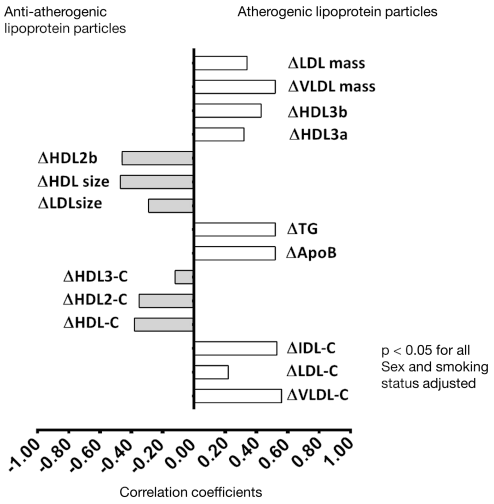

Figure 11 Partial correlates between within-pair difference (Δ) in BMI and lipoprotein profile in monozygotic twins (Study II)

The positive correlations between Δia fat, Δliver fat, and ΔNMR lipoprotein measures associated with atherogenecity (ΔLDL particle concentrations, ΔLDL-C, ΔApoB, ΔApoB-to-ApoA1 -ratio, ΔHDL diameter) were indeed stronger than the correlations between atherogenic of lipid variables and ia and liver fat when twin subjects were analysed as individuals. Spearman correlation analyses using differences within BMI-discordant twin

pairs allows the full control of genetic factors and this indicates that acquired obesity indeed induces pro-atherogenic alterations in lipoproteins independent of genetic background.

The gynoid distribution of body fat associated with anti-atherogenic lipid and lipoprotein measures (Study III). Unlike other fat distribution measures, gynoid fat failed to correlate with atherogenic lipid measures. The positive correlation was strongest between gynoid fat and HDL diameter (r=0.41), negative correlation with medium and small VLDL particles and triglycerides (r=-0.43–0.48). The contribution of lifestyle factors on lipoprotein measures was assessed in study II. The correlation between Δphysical activity and Δatherogenic lipid profile was inverse.

The measures of adiposity are highly inter-correlated: BMI, sc and ia fat more strongly with each other than liver fat. Therefore, in Study II multivariate regression analyses were used to assess if any component of body fat distribution independently explained the within-pair differences in the pro-atherogenic lipid profiles. When entering Δsc fat, Δia fat, Δliver fat, sex, Δsmoking status, and Δphysical activity as independent variables in the models, Δliver fat was the only variable independently explaining the variation in ΔApoB (β=2.2±1.0, p=0.05; whole model adjusted R^2=0.45, p=0.01), ΔTC (β=3.6±1.4, p=0.02; R^2=0.37, p=0.04), ΔLDL-C (β=2.9±1.3, p=0.04; R^2=0.56, p=0.003), ΔHDL3-C (β=0.7 ± 0.3, p=0.04; R^2=0.08, p=0.32;) and also marginally ΔLDL mass (β=5.7±2.9, p=0.07; R^2=0.48, p=0.01). ΔPhysical activity remained significant in models explaining ΔLDL-C (β=-10.4±2.8, p=0.002; R^2=0.56, p=0.003), ΔLDL mass (β=-16.7 ± 6.2, p=0.016; R^2=0.48, p=0.01) and marginally ΔHDL3b (β=-1.5 ± 0.7, p=0.06; R^2=0.28, p=0.088).

5.3.4 The role of fatty liver (Study II)

The results from the analyses within pairs indicate the excess adiposity and the fat distribution modifies the concentrations of lipoproteins towards proatherogenicity. Despite marked differences in weight within pairs (mean Δweight 17kg) the data showed that not all obese co-twin subjects accumulate liver fat. Seven BMI-discordant pairs who did not differ in their liver fat content were identified (Study II). Interestingly, within these liver fat-concordant pairs, all lipid and lipoprotein measures between leaner and heavier co-twins were similar. In contrast, the heavier co-twins with concomitant increase in liver fat (Δliver fat > 2%) had higher levels of ApoB, TC, LDL-C, IDL-C, and HDL3c, higher LDL mass, and lower levels of HDL-C, smaller HDL mean particle size ($p<0.05$) and marginally higher TG concentrations (p=0.07) than their leaner counterparts. In conclusion, proatherogenic lipid derangements emerge only when obesity was accompanied with ectopic fat in the liver.

5.3.5 The insulin resistance (IR) and NMR lipoprotein subfractions (Study III)

In twin individuals, the measures of IR (fasting plasma insulin and HOMA-IR) correlated positively with pro-atherogenic NMR lipoprotein subclasses. Inverse correlations were seen between IR and LDL, and HDL particle diameters, and the ratio of HDL-to-LDL-C. Within MZ twin pairs, Δinsulin resistance correlated positively with ΔTGs and ΔVLDL particle concentrations, Δmedium and Δsmall LDL particles, and ΔApoB. The Spearman correlations between ΔIR and Δlipid measures within pairs were lower compared to the univariate correlation analyses in twin individuals. This is indicative of the genetic control over lipoprotein metabolism in obesity.

5.4 Fatty acids and other NMR metabolites as biomarkers in acquired obesity (Study III)

From NMR small circulating metabolites, the concentrations of branched-chain amino-acids (BCAAs) leucine, isoleucine and valine, as well as lactate, glycerol, urea and inflammatory marker α1-glycoprotein were higher in the heavier co-twins vs leaner co-twins.

BCAAs and α1-glycoprotein correlated positively and with measures of obesity and insulin resistance and negatively with gynoid fat in twin individuals. The correlation between the measures of adiposity and IR and the ratios of PUFAs and omega-6 FAs to total FAs was negative. Within MZ twin pairs, positive correlation was seen between Δα1-glycoprotein, Δisoleucine, and Δinsulin resistance. The correlations between other ΔNMR measures with Δobesity measures and Δinsulin resistance failed to reach statistical significance. This analysis confirms that α1-glycoprotein and BCAAs are plausible candidates for biomarkers of metabolic complications in obesity. The acquired insulin resistance modifies the levels of these NMR metabolites.

5.5 Genetic and environmental correlations between abdominal obesity and circulating NMR metabolic profile (Study III)

The observed phenotypic correlations between abdominal obesity and NMR metabolites were decomposed into their genetic (r_g) and environmental (r_e) components with bivariate modelling (Study III, see Methods, Figure 7). WC correlated with 50 out of 56 NMR metabolites. In most cases, the contribution of genetic and unique environmental component to overall correlations between abdominal obesity and NMR metabolites were similar.

The genetic correlations between abdominal obesity and NMR metabolites were most significant for HDL-C and HDL particle diameter, serum TGs, the ratio of ApoB to ApoA1, BCAAs, phenylalanine, and α1-glycoprotein. Therefore, the effect of the same genes may contribute to the phenotypic traits of abdominal obesity and NMR profile. The environmental correlations were highest for LDL particle diameter, glycerol, and glutamine. Thus, differences in individual unique lifestyles may modify the concentrations of these measures.

5.6 Up-regulation of early complement system pathways in acquired obesity (Study IV)

To investigate the role of complement-system activity during low-grade inflammation in obese AT, the transcriptome array of the complement-system-related genes from AT and isolated adipocytes was analysed. The complement components in AT biopsy samples was localised with immunohistochemical stain.

The expression levels of the classical and the alternative-pathway related genes correlated significantly and positively (r=0.26–0.69) to obesity measures (sc and ia fat volumes, liver fat) and hsCRP, whereas the correlation between the terminal pathway and the obesity measures and inflammation was negative in twin individuals. Altogether 23 out of 46 complement genes expressed in AT, and 25 out of 45 genes expressed by isolated

adipocytes, differed between BMI-discordant co-twins (Figure 11). The expression levels of the genes of the classical, the lectin, and the alternative pathway were up-regulated and the terminal-pathway-related genes down-regulated in obese co-twins. The up-regulated genes included the genes of several complement regulatory proteins and receptors. The plasma levels of C3a were elevated in obese co-twins, whereas the levels of soluble sC5b-C9, a soluble marker of MAC, were similar within BMI-discordant pairs.

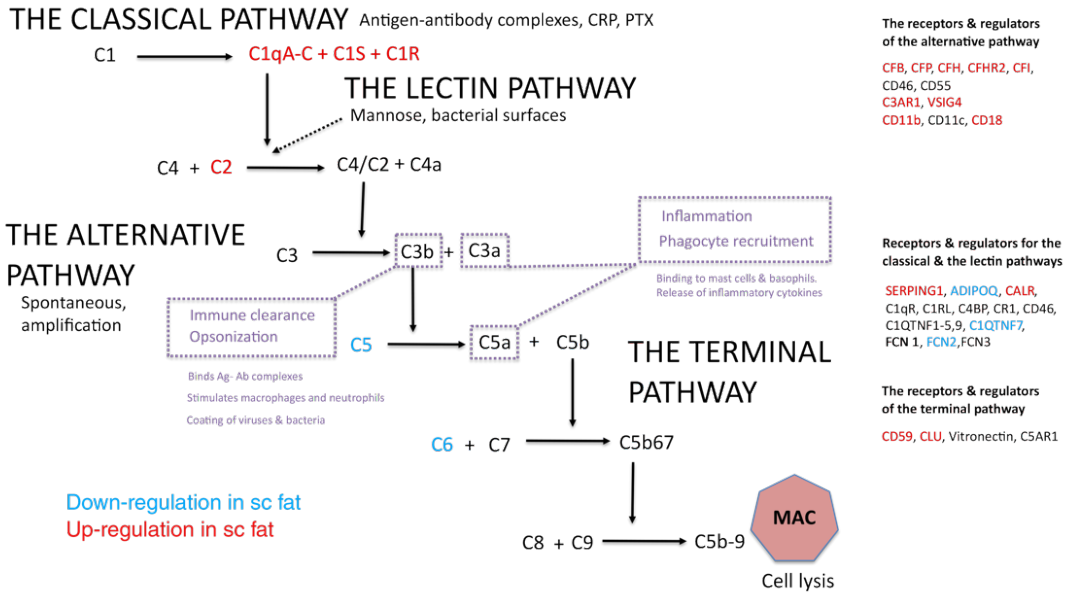

Figure 12 Alterations in complement-system-related genes expressed in sc fat within BMI-discordant co-twins

Modified from Kaye et al. Front Immunol. 2017; 8: 545. doi:10.3389/fimmu.2017.00545

5.6.1 Immunohistochemical stain (Study IV)

Crown-like structures (CLS) represent the dying areas of dying adipocytes. The CLS were more frequent in AT of the heavier co-twins. Two complement components were stained: C1q indicating the initiation of the classical complement pathway; and C3d, a remnant of C3b deposition on cell surface typically observed in prolonged inflammation. C3d stained faintly, and C1q strongly, in adipocyte cell membranes and intracellularly around CLS. Thus, the complement activation is in proximity with the areas enriched with AT immune cells.

5.6.2 Complement gene expression in subcutaneous fat (Study IV)

As the heavier co-twins of BMI-discordant pairs were hyperinsulinemic in comparison to their leaner counterparts (Table 1) we analyzed how the complement gene transcriptome associates with gene expressions of insulin-signalling-pathway related genes. Hyperinsulinemia associated positively with the expression of 9 (out of 15) classical-pathway-related genes in AT ($r=0.28–0.46$, $p<0.03$) and 9/15 genes related to alternative pathway ($r=0.29–0.52$, $p<0.02$). Hyperinsulinemia and the gene expression of C1QTNF7 ($r=-0.36$, $p=0.004$) and the terminal pathway components correlated inversely (C5 r=-0.40 and C6 -0.54). The C5 receptor C5AR1 and clusterin, the inhibitor of MAC complex,

correlated positively to hyperinsulineamia (r=0.44 and 0.66 respectively) in AT. A significant negative correlation emerged between alternative and classical pathway genes and the expression of insulin-signalling-route-related genes in AT.

Correlations between plasma insulin levels and the complement gene expression were not significant for adipocyte transcripts. A significant and negative correlation emerged between the expression of 8/15 classical and 10/15 the alternative pathway related genes and the expression of insulin-signalling-route genes emerged in adipocyte transcripts.

6. DISCUSSION

6.1 The summary of the main results and their interpretation

This thesis arises from twin cohorts in the Finnish population-based registry; one of the largest and most carefully documented twin samples worldwide. The comparison of BMI-discordant young MZ co-twins provides an ideal case-control study setting, fully matching the effect of the genetic background; whereas the adjustment of the genetic background in larger populations is impossible. The results of this thesis complement earlier studies of this Finnish BMI-discordant MZ twin cohort (34)(36)(86)(103)(104) and reveal previously uncharacterised phenotypic differences between obese and lean co-twins.

The MZ twin pairs were already discordant at birth, the heavier co-twin being circa 221g per BMI units (p=0.066 weight in grams, p=0.01 for BMI-unit) heavier in comparison to their leaner counterparts. However, differences in birth weight had disappeared at the age of 6 months but re-emerged after puberty (254). In this study, the acquired obesity was hypothesised to induce differences in metabolism within twin pairs. All metabolic measures were similar within the co-twins of BMI-concordant pairs, indicating strong genetic control of the metabolism. However, obesity-discordant twins differed in their biomarkers of blood coagulation and fibrinolysis, lipoprotein metabolism, and inflammation. Several of these measures have previously been associated with increased risk of thrombosis, cardiovascular disease, and diabetes. Additionally, the obese co-twins were more insulin-resistant and presented more features of low-grade inflammation in comparison to their leaner counterparts. These results conclude the acquired obesity predisposes to hypercoagulable state, increased levels of pro-atherogenic lipoproteins, and activates the complement system in AT. These derangements in metabolism occur already in young otherwise healthy twin subjects without clinical disease. These studies provide new potentially useful biomarkers for characterising an unhealthy metabolic profile in obesity. The accumulation of ectopic fat was a key determinant of these metabolic derangements (Figure 13).

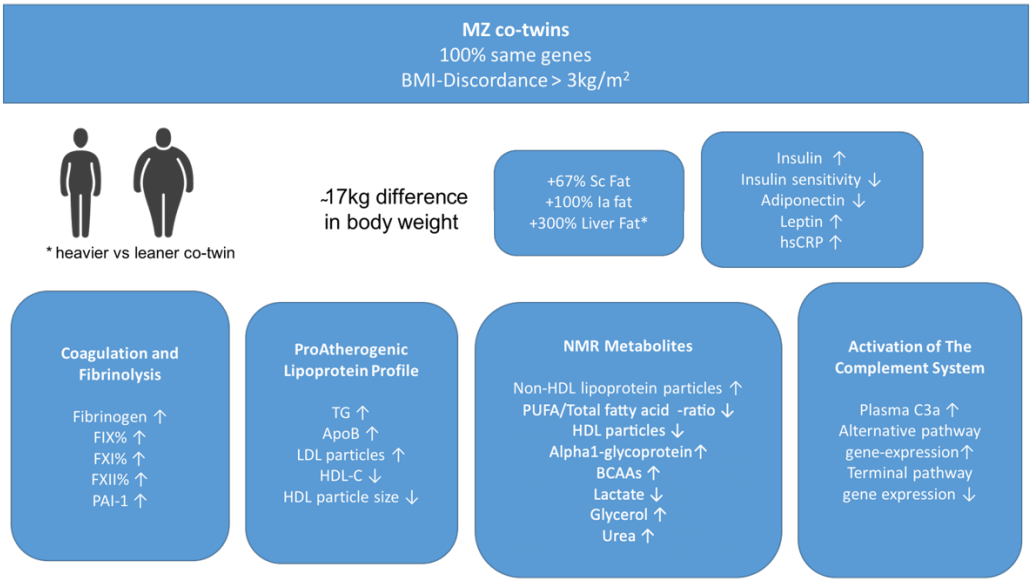

Figure 13 Summary of the metabolic derangements in acquired obesity

In this thesis, the BMI-discordance was defined as within-pair difference (Δ) over 3kg/m^2. A similar definition has been used in other studies (248)(268). To understand the contribution of adipose tissue distribution, the body composition was assessed with DEXA and MRI. These modalities enabled the dissection of fat distribution between the gynoid and android regions, and further division of the abdominal obesity into sc and ia fat compartments. Additionally, proton magnetic spectroscopy was performed to assess liver fat content. Only in study III, including larger number of twin subjects (n=1368), BMI-calculations and waist circumference were collected from the self-reported measurements without imaging. The heavier co-twins of BMI-discordant pairs had substantially higher amounts of body fat. Indeed, the heavier co-twins had nearly 100% more ia fat and up to 280% liver fat compared to their leaner counterparts.

In this study, sc and ia fat correlated the strongest with the derangements of coagulation factors, hyperinsulinemia, pro-atherogenic lipids, and inflammation. The liver fat varied within obese subjects: despite significant within pair differences in body weight within BMI-discordant pairs, not all obese subjects had accumulated liver fat. Fatty liver was shown to be the key driver towards pro-atherogenic lipoprotein profile. The association between unhealthy serum NMR metabolite profile and abdominal obesity was strong. We dissected the correlations between waist circumference and serum metabolite profile into genetic and environmental components. The results demonstrated that both components contribute to the observed associations. Bivariate correlation analyses in the twin cohort revealed that these associations are partly due to shared genetic factors: the same genes control both the waist circumference and the levels of branched-chain amino-acids.

The complement system activation in acquired obesity is present both locally in sc AT and systemically. The gene expression of the early complement pathway was up-regulated and the terminal pathway down-regulated in sc fat microarray. Circulating levels of plasma C3a are higher in obesity. In sc AT biopsies, the complement protein deposits surrounded the apoptotic adipocytes when assessed by immunohistochemical stain.

6.1.1 Overproduction of coagulation factors (Study I)

The genetic factors account for 41–75% of the variation in the levels of coagulation factors (269). As an indication of strong genetic control, the activities of the coagulation factors were similar and highly correlated within BMI-concordant MZ twin pairs. In acquired obesity, the activities of FIX, FXI, FXII, and PAI-1 and the levels of fibrinogen were elevated. As the increase in PAI-1 activity impairs endogenous fibrinolysis, the hypercoagulable state in obesity is due to both increased production of coagulation factors and diminished clot lysis. It is possible that the gender (270), smoking (271), or the use of oral contraceptives (271) may be drivers behind observed changes in coagulation and fibrinolysis. These potential confounders were accounted for by adjusting in the correlation analyses. The effect of leptin, that may promote thrombosis, was not analysed (188). This was the first study showing the magnitude of the effect of acquired obesity on FIX, FXI, and FXII activities. This study affirms that the hypercoagulable and hypofibrinolytic state is an important mechanism behind increased cardiovascular risk in acquired obesity.

Several haemostatic markers are produced in the liver. The fatty liver associates with the overproduction of the coagulation factors (272). The adipocytes secrete pro-inflammatory cytokines that are shown to increase fibrinogen and PAI-1 production in hepatocytes in vitro (166). Interestingly, in the absence of obesity, insulin resistance, and inflammation,

the fatty liver does not fuel the pro-coagulative state (272). In this study within MZ pairs, ΔBMI, Δhyperinsulinemia, and Δinflammation correlated positively with Δfibrinogen and ΔPAI-1 activities. This indicates the independent effect of obesity on coagulation and fibrinolysis. PAI-1 activity is directly correlated to AT mass and its gene-expression is upregulated in VAT (273). The effect of adiposity on blood coagulation appears stable over time as Δfibrinogen levels persist within co-twins in pairs who remain BMI-discordant over time (248).

The results from weight loss studies support the role of acquired obesity on haemostasis. The activity of PAI-1 and fibrinogen levels decrease after weight loss by lifestyle intervention (274). Similarly, the levels of coagulation parameters decreased after bariatric surgery (275). Additionally, as shown in longitudinal follow-up of the MZ twin cohort, before the BMI-discordance within co-twins emerged, fibrinogen levels were similar (248). These findings indicate that adverse changes in blood coagulation are induced by increased adiposity and the pro-thrombotic state alleviates with weight loss.

The adipose tissue adipokines, including leptin, resistin, and visfatin, modulate the pro-thrombotic state (178)(276)(277). The proposed mechanism for adipokine effects is enhanced activation of the expression of pro-thrombotic adhesion molecules in vascular endothelium as demonstrated by resistin and visfatin (178). In contrast, adiponectin and apelin have anti-thrombotic effects (117)(278). As the serum adiponectin levels in the obese twins were decreased vs. leaner co-twins, this may aggravate the pro-thrombotic state in acquired obesity. Additional studies on endothelial function would complement the current data. As the pro-inflammatory environment in obesity can significantly contribute to plasma PAI-1 levels (189) it would be interesting to analyse the role of innate immunity and its association of fibrinolytic measures in acquired obesity.

6.1.2 The role of fatty liver-overproduction of lipoproteins (Study II)

Abdominal obesity and metabolic syndrome are characterised by increased TG-rich lipoprotein particles, formation of small atherogenic LDL-particles, and low concentrations and diminished size of HDL lipoproteins, as well as lower levels of HDL-C. As expected, the obese co-twins in this thesis study had pro-atherogenic lipoprotein profiles, and this was affirmed both with ultracentrifugation (Study II) and later with NMR (Study III). The leaner co-twins presented a healthier lipid and lipoprotein profile. The BMI-concordant MZ twin pairs presenting similar lipid profiles confirm the strong genetic control of lipoprotein metabolism. This is the first study to document that acquired obesity does not significantly alter the lipoprotein particle composition, including surface phospholipids, protein component, and core lipids. Indeed, the derangements in lipoprotein metabolism in acquired obesity are due to changes in the particle numbers.

Unique to this study, two distinctive groups were identified within the heavier co-twins of BMI-discordant pairs. In the other sub-group, acquired obesity was accompanied by a concomitant increase in liver fat, whereas in another subgroup, no difference in liver fat emerged despite a 17kg weight discordance. Liver fat content correlates more significantly within MZ than DZ twin pairs, and the heritability of hepatic steatosis is high (137)(279). The genetic factors may explain how in this study some, but not all, heavier co-twins had elevated liver-fat content despite similar Δweight. Several genetic variants, including those of PNPLA3 and TM6SF2 genes, predict the development of fatty liver in adults (280)(281). As the obesity roots from sedentary lifestyle with overconsumption of indigested calories,

these unique environmental factors may trigger liver fat accumulation in genetically vulnerable individuals.

We discovered that the lipoprotein measurements differed within BMI-discordant pairs only when they were also liver-fat discordant. In unhealthy obesity, the enlarged ia fat volume is likely contribute to derangements of lipoprotein metabolism. However, in this study, out of body fat depots, in multivariate regression analyses, the contribution of liver fat remained significant towards proatherogenic lipid profile. Obese co-twins with fatty liver had elevated TGs, higher ApoB, and LDL-C. This represents the overproduction of TG-rich VLDL particles. Without increase in liver fat, these signs of proatherogenicity did not occur in acquired obesity. ApoB is an essential protein required for assembly and secretion of VLDL from the liver. The elevated ApoB in obese co-twins represents the increased number of both VLDL as well as LDL particles. Consequently, this data showed the VLDL and LDL particle masses were elevated in obesity. Impaired clearance of TG-rich lipoproteins and the complex lipid exchange between lipoprotein particles induces the formation of small particularly atherogenic LDL-particles (146). In acquired obesity, the simultaneous decrease in HDL-particle size and HDL-C associates with impaired reverse cholesterol transport capacity to the liver. This contributes to the formation of cholesterol plaques in vessels and the risk of atherosclerotic vascular disease. This data affirms that these proatherogenic alterations occur before the manifestation of clinical disease in young adults.

Recently, a twin study by Cui et al (282) revealed shared genetic factors behind hepatic steatosis, elevated TGs, and low HDL. Furthermore, insulin resistance and hyperglycaemia shared common genetic pathways with fatty liver. In line with Cui, in this study data Δpro-atherogenic lipoprotein measures associated significantly to the Δmeasures of systemic insulin resistance within MZ twin pairs. It is well-known that IR and dyslipidaemia cluster in the metabolic syndrome and the data supports the underlying common genetic pathways behind phenotypic traits.

The dysfunctional AT with limited capacity to expand is contributing to liver fat accumulation and pro-atherogenic lipoprotein profile. Limited expansion in adipocyte cell volume and the compromised recruitment of new adipocytes for lipid storage leads to ectopic fat distribution outside AT, also into the hepatocytes. Heinonen et al. (86) reported that the sc AT morphology was altered in acquired obesity. The AT of obese co-twins was indeed hypertrophic and hypoplastic in comparison to their leaner counterparts. In obese AT, the adipocyte hypertrophy, instead of adipocyte hyperplasia, associated with impaired insulin signalling and AT insulin resistance (86). The impaired insulin action leads to uncontrollable lipolysis from AT. The fatty acid flux from lipolytic AT provides substrate for hepatic TG accumulation contributing to increased concentrations of plasma TG-rich lipoproteins as demonstrated in these studies. Therefore, the compromised capacity of sc AT capacity to expand and store lipids is one plausible mechanism behind pro- atherogenic lipoprotein metabolism documented in this study.

The overconsumption of calories, high intake of simple sugars (283) or saturated fats can increase the hepatic fat content (284). In contrast, the exercise training reduces the liver fat also independent of diet (67)(285). As underlying genetic factors may also contribute to the response of body composition following physical activity (286), the combination of diet and exercise is useful to control both the fat accumulation to the liver and plasma lipid abnormalities (287). In study II, physical activity was identified as an independent predictor

for healthy lipid profile. It is reasonable to assume that exercise improved the insulin sensitivity in physically active twins. Consequently, the improved insulin action controls sc AT lipolysis and lipid spill to ectopic tissues, including the liver. Further studies on twin pairs discordant in regard to physical activity would provide insight into the potential underlying mechanisms of exercise on lipoprotein metabolism.

A subgroup of obese subjects does not seem to develop obesity-related metabolic complications, at least in the early phase of obesity. The key characteristics of metabolically healthy obesity (MHO) are the lack of features of MetS, low liver fat content, high insulin sensitivity and aerobic fitness, preserved mitochondrial function and low systemic inflammation despite high BMI (37)(288)(289). The prevalence of MHO varies depending on definition of MHO and cohort. In the Finnish population cohort, the prevalence was 23% (290). However, the lack of a uniform definition of MHO is a limitation for characterisation of the subjects in practice. Age, sex, cardiorespiratory fitness, diet, and ethnicity are known contributors to metabolic status but often they are not taken account of in the assessment of metabolic health (291). Longitudinally, the conversion towards obesity-associated metabolic complications implicates also that MHO is not a stable condition. After 5 years of follow-up, 35% of originally MHO individuals had developed metabolic derangements and only 10% were still metabolically healthy after 20 years (292). Thus, cross-sectional studies do not predict the future health risk in MHO.

The VAT accumulation, adipose tissue morphology and function contribute to obese, but metabolically healthy phenotype (291). In BMI-discordant (within pair difference Δ18kg) MZ twin pairs, the excess liver fat dichotomised metabolically healthy and unhealthy groups (37). Low inflammation gene transcripts and preserved mitochondrial function characterised the sc fat of the MHO co-twins (37). These features potentially reflect better fat storing capacity in sc AT and protection from ectopic fat accumulation viscerally and in the liver. MHO subjects seemingly adapt to overnutrition and surplus of energy. They present similar glucose and insulin responses after high-calorie meals as lean individuals (293). After a meal, preserved postprandial incretin response associates to healthy obesity and may reflect better beta-cell function and the capacity to increase insulin secretion from beta-cells (294).

6.1.3 Serum metabolomics (Study III)

New risk markers have been identified from serum metabolomic profiles for prediction of CVD (295) and insulin resistance (296) and even all-cause mortality in obese subjects (297). In the present study, the levels of BCAAs, as well as pro-atherogenic lipids and α1-glycoprotein, were elevated in acquired obesity. No differences in NMR metabolite panel within BMI-concordant pairs emerged. In a study utilising Mendelian randomisation, adiposity was shown to directly alter the levels of multiple cardiometabolic risk markers in young adults (298).

In the correlation analyses in twin individuals, we found out that the android/abdominal fat distribution associated with high-risk metabolites, whereas the association of the gynoid fat distribution with metabolomics signature was more favourable. The abdominal fat distribution correlated strongly and positively with pro-atherogenic lipid profile, increased levels of BCAAs, and α1-glycoprotein, and negatively with glutamine. Both environmental and genetic factors contribute to the observed correlations. For ApoA1, BCAA, and phenylalanine, the contribution of genetic correlation (r_g= -0.24, 0.32, and 0.36

respectively) was significant and stronger than the contribution of unique environmental factors to the co-occurrence of the abdominal obesity and metabolite profile. This study data suggests that shared genes determine both phenotypes: abdominal obesity and serum levels of metabolites.

BCAAs are catabolised in mitochondria and their oxidative capacity determines the rate of this process. In the BMI-discordant twin cohort, as well as in other studies, the mitochondrial number in sc AT is reduced and their functional capacity is impaired in acquired obesity (103)(299)(300). Thus, mitochondrial dysfunction in sc AT may explain the observed differences in BCAA metabolism in acquired obesity, even though the role of abundant mitochondria in muscle or BCAA catabolism liver were not analysed. BCAA metabolism may have a causal role in the development of type 2 diabetes in insulin-resistant obesity (301)(302).Yet, all studies do not support this hypothesis (303)(304) and more research is needed. The link between mitochondrial function and IR appears to be bidirectional (300). The reactive oxygen species and inflammatory response contribute to the underlying metabolic pathways and cannot be disregarded (300).

6.1.4 The complement system in obesity (Study IV)

The active role of AT and adipocytes in cross-talk with the immune system is notable. This study indicates the interplay of complement system activity and AT, and contributes to the AT dysfunction in obesity. Highlighting the role of gene-environment interactions and the heritability of the complement component levels (305), this study elucidates the contribution of the unique lifestyle on complement system activity. The differences within MZ co-twins emerged only if they were discordant on obesity. In agreement with the earlier twin study of di Franco et al showing C4 and factor B levels are modifiable by environmental exposures (306), this study's co-twin comparison of obesity-discordance showed obesity does induce differences in the activity of the complement system. The abdominal obesity in particular associated with the activation of complement system and a similar conclusion has been made in other studies (307)(308).

The activation of the complement system occurs both locally in sc AT systemically as plasma levels of C3a and adipsin increase. The complement component C3 is a well-known biomarker for the risk of CVD (222). Indeed, C3 and its metabolites are present in plasma thrombus and have anti-fibrinolytic properties (309)(310). Local complement activation was seen in sc fat as early complement pathway gene expression was up-regulated in AT and adipocyte gene transcripts. In accordance with these results, the expression of complement-system-related genes by adipocytes has been documented elsewhere (222). In obesity, the gene expression of the terminal pathway was suppressed in sc fat. This indicates that rather than being involved in cell destruction and tissue damage, the role of complement is the opsonisation clearance of debris in AT. The role of complement regulators controlling the activation of the terminal pathway is crucial maintaining the tissue integrity. Indeed, the gene-expression of clusterin, an inhibitor of the terminal pathway significantly up-regulated in obese co-twins of the BMI-discordant pairs and correlated strongly with all obesity measures. The results are in line with Won et al describing the role of clusterin in AT inflammation (311).

Adipocyte hyperplasia is linked to increased rate of apoptosis (312). Apoptotic cell death and subsequent macrophage infiltration in AT is a link to detrimental metabolic sequalae, IR, and ectopic fat deposition in obesity (313). In histochemical stains, the complement

components were localised in sc AT biopsies in macrophage-rich areas surrounding dying apoptotic cells. In adipocytes, functional insulin signalling allows the regulation of glucose and FA uptake. In obese sc AT, the up-regulation of complement gene expression in adipocytes negatively associated with the expression of the genes related to the insulin signalling route. Therefore, the complement system may play a role in the development of the AT IR. The mechanisms that initiate the apoptosis are not yet understood. Concomitant mitochondrial dysfunction and lack of capacity to produce energy for metabolic needs may act as a trigger for the terminal process of programmed cell death.

6.2 Limitations

BMI-discordance in MZ twins is extremely rare. Only 26 healthy pairs were identified from 10 Finnish yearly population-based birth cohorts. No other case-control study setting has facilitated as careful a match for genetic and other shared environmental factors. Despite a relatively low number of subjects, these studies still capture meaningful differences in metabolism in acquired obesity. Twin studies have a key role in determining the magnitude of genetic and environmental contribution to individual differences for obesity-related metabolic derangements. Combining twin registries adds power to studies in future obesity-discordant twin studies (314).

In study III, only self-reported obesity measures were collected from the larger twin cohort. The intensively studied, obesity-discordant twin pairs and their controls were exposed to physical examinations, numerous biochemical analyses, radiological examination, and invasive AT biopsies. The demanding study protocol may have limited the number of consenting participants. The data on BMI-discordant pairs declining the invitation for these studies was not available when summarising this thesis. The unique differences on the physical activity and diet were documented in food and exercise diaries from participants but only in the study II this data was analysed. The magnitude of the effect of the differences in these lifestyle factors on metabolism was beyond the scope of this thesis.

Study III reports only the selected metabolites (n=56) from NMR. The results from the full NMR metabolomic panel are reported in other studies (315). For study IV, total RNA was extracted from whole-adipose tissue biopsies and transcriptomics analyses of fat were performed by microarray. Microarray probe design limits the identification of complement-protein related genes in sc AT biopsies. Currently, more developed high-throughput sequencing techniques enable quantifying the transcriptomes with more precision (316).

The setting of studies I-IV is cross-sectional and describes the nature of the metabolic derangements ‘here and now’. As discordance in MZ twins is rare and may be only temporary (248), the differences in metabolism may not persist over time. Additionally, as the observed differences in metabolism within discordant pairs are subtle, the clinical relevance of acquired obesity in young adulthood over individuals’ lifespan is unknown. Even if obese co-twins presented with an unhealthy metabolism at the time of the data collection for studies I-IV, the increase in morbidity cannot be predicted without prospective follow-up. The follow-up data would elucidate the burden or legacy of acquired obesity.

7. CONCLUSIONS AND FUTURE PROSPECTS

Obesity-related metabolic disturbances arise from complex gene-environment interplay. Overall similarity within MZ pairs indicates the strong genetic control of metabolism. Yet, the differences in metabolism between obesity-discordant co-twins arises from the differences in unique lifestyles. This thesis describes how acquired obesity associates with unhealthy metabolism. The derangements of metabolism are subtle but widespread and correspond to several metabolic pathways predisposing to CVD. Indeed, the risk factors for atherosclerosis, thrombosis, and diabetes cluster in acquired obesity irrespective of genetic background. The signature of unhealthy metabolism manifests already early in young adulthood, which emphasises the importance of adopting healthy lifestyle and balanced energy intake already at an early age.

It is important to note that the metabolic risk varies even within the same obesity class. Body fat distribution and adipose tissue function determine individual metabolic responses and healthier features, including low levels of proatherogenic lipids, coagulation factors, and inflammation, associated with lower levels of liver and ia fat. The metabolic risk should always be assessed individually. A combined measure of BMI and a radiological measure of ia or ectopic fat would help to distinguish the individuals with the highest risk. Based on the analyses in this thesis, liver fat associates with derangements in lipid metabolism. This implies new therapeutic targets towards dyslipidemia may arise from molecules that modify liver fat content. As hypercoagulable state and the complement activation co-occur in obesity, the complement system may provide new therapeutic targets for atherothrombosis and controlling the low-grade inflammation in obese subjects. When lipids are stored in sc AT adipocytes, the lipolysis is controlled, AT can expand, and the liver and other organs are protected from ectopic lipid accumulation. Better understanding of the adipocyte biology, characteristics of energy storing, and dispersion properties may eventually offer a measure of the energy deposition threshold in AT before lipid spillover occurs.

The high prevalence of obesity-related morbidity is a burden and challenging the global health care resources. Overall obesity increases morbidity and even mortality. With current understanding, defining the healthy level of overweight or obesity is impossible. Obese individuals carrying the highest metabolic risk need support and resources for lifestyle and pharmacological interventions, whereas when metabolism remains healthy, less intensive monitoring is needed. Nevertheless, for all individuals, regardless of their obesity status, it is important to support the efforts of preserving healthy lifestyle to prevent further weight gain.

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APPENDIX

Appendix Table 1 Summary of available clinical and anthropometric measures in Studies I-IV

	Study I	Study II	Study III			Study IV
			Genetic correlation analyses	Correlation analyses	Within pair analyses	
BMI	x	x	x	x	x	x
Waist	x	x	x	x	x	x
DEXA	x	x	x	x	x	x
MRI	x	x	x	x	x*	x
MRS	x	x	x	x	x*	x
Fasting blood sample	x	x	x	x	x	x
Insulin	x	x	x	x	x	x
Euglycemic hyperinsulinemic clamp	x					
Glucose	x	x	x	x	x	x
hsCRP	x	x	x	x	x	x
Adipose tissue biopsies	x					x
Lifestyle questionnaires	x	x	x	x	x	x

*84 individuals

Appendix Table 2 Summary of the laboratory analyses from plasma/serum

	Laboratory measure	Assay Kit/Method	Manufacturer
Plasma Coagulation Factors (Study I)			
	PT	Nycotest® PT reagent	Axis-Shield PoC AS, Oslo, Norway
	APTT	Actin® FSL reagent	Siemens Healthcare Diagnostics
	Fibrinogen	Multifibren®	
	VWF ristocetin cofactor (VWF:RCo)	BC® von Willebrand Reagent	
	FVII	Dade® Innovin® and Coagulation Factor VII Deficient Plasma	
	FVIII, FIX, FXI, FXII	Pathromtin SL®, and specific coagulation factor deficient plasma	
	FXIII	Berichrom®	
	D-dimer	Tina-quant D-Dimer®,	
	PAI-1	Asserachrom PAI-1, Chromolize PAI-1	Diagnostica Stago, Gennevilliers,France Biopool International, Umeå, Sweden
Serum Lipoprotein Measures (Study II)			
	Total Cholesterol	Enzymatic method, Konelab 60i analyzer	Thermo Fisher Scientific Oy
	Triglycerides		
	ApoA1	Immunoturbidometry	Wako Chemicals GmbH, Neuss, Germany
	ApoB		Orion Diagnostica, Espoo, Finland
	ApoC3	ELISA	Ref (317)
	VLDL, IDL, LDL, HDL2 and HDL3	Ultracentrifugation	Ref (318)
	Lipoprotein compositions	Enzymatic Konelab 60i analyzer	Thermo Fisher Scientific Oy
	HDL 2b, 2a, 3a, 3b, and 3c subspecies and HDL mean particle size	Gradient gel electrophoresis	Ref (319)(320)
	Mean HDL particle size	calculation from the area under the densitometric scan	
	Mean LDL particle size		Ref (321)
Serum adipokines (Study IV)			
	Adiponectin, Adipsin	DuoSet ELISA	R&D Systems Europe Ltd, Abindgon, UK
Metabolomics (Study III)			
	NMR metabolites	NMR Spectroscopy	Ref (263), (262)
Complement components (Study IV)			
	C3a	Enzyme-linked immunosorbent assay, MicroVue C3a Plus	Quidel Corporation, San Diego, CA, USA
	Soluble C5b-9 terminal pathway complex	C5b-9 Plus enzyme immunoassay (EIA)	Quidel Corporation, San Diego, CA, USA

Original Publications

Obesity-Related Derangements of Coagulation and Fibrinolysis: A Study of Obesity-Discordant Monozygotic Twin Pairs

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Coagulation and fibrinolytic activities are under strong genetic control. We studied the effects of acquired obesity, independent of genetic factors on coagulation and fibrinolysis activities in obesity-discordant healthy monozygotic (MZ) twin pairs. Fourteen obesity-discordant (BMI within-pair difference >3 kg/m^2) and 10 concordant (BMI difference <2 kg/m^2) MZ twin pairs were identified from the nationwide FinnTwin16 study. Body composition (dual-energy x-ray absorptiometry), abdominal fat distribution (magnetic resonance imaging), liver fat (magnetic resonance spectroscopy), high sensitivity C-reactive protein, insulin sensitivity (euglycemic hyperinsulinemic clamp), and a panel of different markers of blood coagulation and fibrinolysis in the fasting state were measured. Strong resemblance was observed in most coagulation factors within all twin pairs, with the intraclass correlations ranging from 0.73 to 0.97, $P < 0.03$. However, the activities of fibrinogen and FIX, FXI, and FXII, and plasminogen activator inhibitor-1 (PAI-1) activities were increased in the obese co-twins ($P < 0.05$) and strongly correlated with the measures of adiposity, inflammation, and insulin resistance ($r = 0.32–0.73$, $P < 0.05$) among the twin individuals. Intrapair differences in fibrinogen and PAI-1 correlated with those in BMI, adiposity, and fasting insulin levels ($r = 0.40–0.58$, $P < 0.05$) indicating the independent effect of obesity. Derangements of blood coagulation and fibrinolysis are present already in early adulthood in obese subjects. Acquired obesity, independent of genetic factors, increases the activities of fibrinogen and activities of FIX, FXI, FXII, and PAI-1. This study confirms the mechanisms of simultaneous activities of intrinsic coagulation factors and impaired fibrinolysis predisposing obese subjects to thrombosis.

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INTRODUCTION

Obesity is characterized by multiple hemostatic disturbances in blood coagulation, including enhanced platelet activation (1), increased concentrations, and enhanced activities of plasma coagulation factors (2–4) as well as impaired fibrinolysis in form of increased production of plasminogen activator inhibitor-1 (PAI-1) (5). Several other mechanisms, such as systemic inflammation (6), endothelial dysfunction, disturbances of lipid and glucose metabolism, and insulin resistance also contribute to the hypercoagulable state in obesity (7).

Both environmental (8,9) and genetic factors account for the altered coagulation profile in obese individuals. Genetic factors contribute to the interindividual variation of blood coagulation protein levels (10–12). Inherited genetic polymorphisms influence the hemostatic profile and increased risk of vascular events (13). Pleiotropically acting genes may contribute to the clustering of procoagulant and metabolic risk factors in obese individuals (10,14,15).

In study settings investigating the relationship between obesity and coagulation, it is difficult to disentangle the influence of the genetic background from the effects of acquired obesity *per se*. Monozygotic (MZ) twin pairs discordant for obesity offer a unique opportunity in humans to study the effect of obesity on coagulation and fibrinolysis while controlling the genetic background. The aim of our study was to investigate how obesity, independent of genetic factors, influences markers of coagulation and fibrinolysis. We chose a set of coagulation tests which reflect established risk factors for thrombosis and analyzed their possible links with various markers of adiposity, inflammation, and insulin sensitivity.

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METHODS AND PROCEDURES

Subjects

The participants belong to the FinnTwin16 cohort, a population-based, longitudinal study of five consecutive birth cohorts (1975–1979) of twins, their siblings and parents (16). Twin pairs were recruited to the current study based on their responses to questions on weight and height in a questionnaire (response rate 88%) at the age of 23–27 years (17–19). After screening all MZ twin pairs (n = 658), we identified 18 pairs with a reported BMI difference of at least 4 kg/m^2 (MZ discordant pairs), such that one co-twin was nonobese (mean BMI ≈25 kg/m^2), whereas the other was obese (mean BMI ≈30 kg/m^2) without having associated comorbidities. Fourteen of these pairs (eight male and six female pairs) participated. In addition to these weight-discordant pairs, we studied 10 concordant MZ pairs (five male and five female pairs) with an intrapair BMI difference of less than 2 kg/m^2.

The subjects were healthy, normotensive and did not use any medications, except for oral contraceptives (n = 8 of 22 women). Their weight had been stable for at least 3 months before the study. Females were scheduled to attend during the follicular phase of their menstrual cycle. Only four of the 48 participants were currently smokers. Two pairs had a family history of type 2 diabetes. Monozygosity was confirmed by genotyping 10 informative genetic markers (18). The subjects provided written informed consent. The protocol was designed and performed according to the principles of the Helsinki Declaration and was approved by the ethics committee of the Helsinki University Central Hospital. Detailed description of the MZ twin material has been published earlier (17,20).

Clinical assessment

Body composition was measured by dual-energy x-ray absorptiometry (Lunar Prodigy, software version 2.15; GE Healthcare, Madison, WI) (21). Subcutaneous and intra-abdominal fat were determined by magnetic resonance imaging of 16 transaxial scans reaching from 8 cm above to 8 cm below the fourth and fifth lumbar interspace (17). Liver fat content was measured by proton magnetic resonance spectroscopy (22). This measurement has been validated against histologically determined lipid content in our laboratory (23) and against estimates of fatty degeneration or infiltration by X-ray computer-assisted tomography (22). All spectra were analyzed by a physicist in a blinded fashion. The reproducibility of repeated measurements of liver fat in nondiabetic subjects was 11% when studied on two occasions in our laboratory (24).

Insulin sensitivity

Whole body insulin sensitivity was determined by the euglycemic hyperinsulinemic clamp technique (25). Two 18-gauge catheters (Venflon; Viggo-Spectramed, Helsingborg, Sweden) were inserted, one in an antecubital vein for infusion of insulin and glucose, and another retrogradely in a warmed hand vein to obtain arterialized venous blood for measurement of plasma glucose concentrations every 5 min and serum free insulin concentration every 30 min. Regular human insulin (Insulin Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was infused in a primed-continuous fashion. The rate of the continuous insulin infusion was 40 mU/m^2/min (1 mU/kg/min) for 120 min. Normoglycemia was maintained by adjusting the rate of a 20% glucose infusion based on plasma glucose measurements from arterialized venous blood every 5 min. Whole body insulin sensitivity (the M-value, expressed as mg/kg fat-free mass/min) was determined from the glucose infusion rate needed to maintain normoglycemia after correction for changes in the glucose pool size (25). Because hepatic glucose production is maximally suppressed in nondiabetic subjects already at an insulin concentration achieved during infusion of insulin at a rate of 0.5 mU/kg/min (26), the M-value reflects the glucose uptake.

Clinical chemistry

Blood samples were collected from antecubital veins at 8:00–8:30 AM after a 10–12 h overnight fast. Plasma and serum were separated by centrifugation for 15 min at 2,000g at 4 °C. The separated plasma and serum were frozen in aliquots at −70 °C until assayed. Samples from tubes containing 0.109 mol trisodium citrate were analyzed for prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen, and for activities of von Willebrand factor (VWF), FVII, FVIII, FIX, FXI, FXII, and FXIII by using the BCS XP coagulation analyser (Siemens Healthcare Diagnostics, Marburg, Germany) at the coagulation laboratory of the Helsinki University Hospital (HUSLAB). PT was measured by using Nycotest PT reagent (Axis-Shield, Oslo, Norway), APTT by Actin FSL reagent (Siemens Healthcare Diagnostics), and fibrinogen with a modification of the Clauss method (Multifibren U; Siemens Healthcare Diagnostics). D-dimer was assessed with the immunoturbidimetric assay (Tina-quant D-Dimer; Roche Diagnostics, Mannheim, Germany). For activities of VWF ristocetin cofactor (VWF:RCo), we used the BC von Willebrand reagent, for FVII Dade Innovin and coagulation factor VII deficient plasma and for FXIII Berichrom FXIII, all the reagents from Siemens Healthcare Diagnostics. For one-stage FVIII, FIX, FXI, and FXII activity measurements we used Pathromtin SL and the specific coagulation factor deficient plasma, respectively (all from Siemens Healthcare Diagnostics). The local reference ranges for the coagulation variables were as follows: PT 70–130%, APTT 23–33 s, fibrinogen 1.7–4.0 g/l, D-dimer <0.5 mg/l, VWF:RCo 44–183%, FVII 76–170%, FVIII 52–148%, FIX 67–135%, FXI 60–120%, FXII 60–150%, and FXIII 76–156%.

Serum high sensitivity C-reactive protein (hsCRP) was measured using the double antibody sandwich enzyme-linked immunosorbent assay with rabbit antihuman CRP and peroxidase-conjugated rabbit antihuman CRP. The assay was linear up to 5 mg/l and logarithmic thereafter. Plasma PAI-1 activity was assayed with commercially available kits (Asserachrom PAI-1; Diagnostica Stago, Gennevilliers,France and Chromolize PAI-1; Biopool International, Umeå, Sweden respectively). The local reference values for PAI-1 activity were 7 (3–17) IU/ml (median (interquartile range)). One unit of PAI-1 activity is defined as the amount of PAI-1 that inhibits one international unit of human single chain tissue plasminogen activator as calibrated against the International Standard for tissue plasminogen activator, lot 86/670 distributed by National Institute for Biological Standards and Control, Holly Hill, England. PAI-1 activity was measured in 32 MZ individuals, 16 full pairs and hsCRP in 40 individuals, 19 full pairs. Overall, the inter-assay and intra-assay coefficients of variation were 10% or less across the measurements of all variables.

Statistical analyses

The statistical analyses were performed using the Stata statistical software (release 9.0; Stata, College Station, Texas). Survey data procedures were used to correct for clustered sampling of co-twins within pairs (27). Log-transformation was used to normalize the distribution of non-normally distributed data. Wald tests for independent samples (=t-tests adapted for clustered twin data) were used to compare men vs. women, users vs. nonusers of contraceptives, and smokers vs. nonsmokers. Pearson correlations, with correction for clustering (27), were calculated to determine the relationships between clinical and biochemical parameters in individual twins. The co-twins were compared by Wilcoxon's signed ranks test. Male and female pairs were combined because by definition, MZ co-twins are matched for gender. Spearman correlations of within-pair differences in body composition and metabolic measures were used to test the effects of the extent of adiposity discordance on coagulation. Because MZ twins are identical at the sequence level and share all their segregating genes, the associations within MZ pairs are fully adjusted for genetic effects. Twin similarity was assessed using intraclass correlations.

RESULTS

Body composition and insulin resistance in twin pairs

The characteristics of MZ twins examined in the present study are summarized in **Table 1**. The intrapair differences of BMI ranged from 0.0–2.3 kg/m^2 in the weight-concordant pairs and from 3.8–10.1 kg/m^2 in the discordant pairs. Concordant MZ

Table 1 Physical characteristics and levels of coagulation factors and variables measuring fibrinolytic activity in monozygotic (MZ) twins

	MZ concordant *n* = 10 pairs		MZ discordant *n* = 14 pairs	
	Leaner	Heavier	Leaner	Heavier
BMI (kg/m^2)	26.3 ± 2.3	27.3 ± 2.3**	25.3 ± 0.5	30.5 ± 0.5***
Body fat %	25.2 ± 3.9	27.5 ± 4.0*	29.7 ± 2.4	40.2 ± 2.1***
Subcutaneous fat (dm^3)	2.7 (1.4–3.8)	2.9 (1.6–4.2)	2.7 (2.4–4.0)	4.8 (4.4–5.8)***
Intra-abdominal fat (dm^3)	0.6 (0.4–0.9)	0.6 (0.4–1.3)	0.5 (0.4–0.7)	1.0 (0.8–1.2)***
Liver fat (%)	1.5 (1–7.5)	1 (1–9)	1.25 (1–2.5)	3.6 (2–11)***
M-value (mg/kg fat-free mass/min)	7.4 ± 0.9	7.5 ± 1.0	9.2 ± 0.9	6.3 ± 0.6**
Insulin (mU/l)	6 (5–9)	6.5 (4–10)	5 (3–8)	9 (6–11)*
hsCRP[a] (mg/l)	0.5 (0.1–1.4)	0.5 (0.2–4.0)	0.9 (0.2–1.1)	2.0 (0.9–3.4)**
Fibrinogen (g/l)	3.2 (2.7–3.7)	2.9 (2.6–4.0)	3.2 (2.4–3.5)	3.4 (2.8–4)**
FVII (%)	110 ± 7.8	106 ± 5.4	118 ± 5.8	117 ± 5.6
FVIII (%)	94 ± 9.8	93 ± 11.4	97 ± 6.5	101 ± 6.7
FIX (%)	95 ± 4.7	96 ± 6.2	95 ± 3.8	105 ± 3.8*
FXI (%)	91 ± 4.4	93 ± 5.6	96 ± 3.8	105 ± 4.8*
FXII (%)	96 ± 6.9	95 ± 6.4	100 ± 6.5	108 ± 7.0**
FXIII (%)	87 ± 5.0	86 ± 4.9	83 ± 5.4	86 ± 5.3
VWF:RCo (%)	70 (57–102)	64 (57–84)	89 (81–128)	93 (63–101)
PAI-1[b] (%)	1.1 (0.0–4.0)	1.4 (0.7–5.8)	4.1 (3.4–21.2)	17.5 (8.3–25.1)**
D-dimer (mg/l)	0.05 (0.05–0.1)	0.05 (0.05–0.1)	0.05 (0.05–0.1)	0.05 (0.05–0.1)

Data are mean ± s.e., or for non-normally distributed variables and median followed by the 25th and 75th percentiles. Wilcoxon's test for paired samples, leaner vs. heavier co-twin, *P* values adjusted for sex, smoking, and use of oral contraceptives.
F, coagulation factor; hsCRP, high sensitivity C-reactive protein; MZ, monozygotic; PAI-1, plasminogen activator inhibitor-1; VWF:RCo, von Willebrand factor ristocetin cofactor activity.
[a]11 MZ discordant pairs, 9 MZ concordant pairs. [b]9 MZ discordant pairs, 7 MZ concordant pairs.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

co-twins did not differ in measures of fat distribution (magnetic resonance imaging), insulin sensitivity (M-value, fasting glucose or insulin) or inflammation (hsCRP). In the discordant pairs, the heavier co-twins had 1.8 times the amount of abdominal subcutaneous fat, 2.0 times the amount of intra-abdominal fat, and 2.9 times the amount of liver fat compared with their leaner counterparts. The heavier co-twins also had lower insulin sensitivity and higher fasting insulin levels compared with the leaner co-twins. hsCRP values were over twofold higher in the heavier than in the leaner discordant co-twins.

Measures of coagulation factors and fibrinolysis in twin pairs

Each coagulation factor was first tested with respect to differences between genders, users and nonusers of contraceptives, and between smokers and nonsmokers. Men had lower levels of fibrinogen (2.9 vs. 4.0 g/l, $P = 0.0006$), D-dimer (0.08 vs. 0.11 mg/l, $P = 0.025$), lower activities of FVII (106 vs. 123%, $P = 0.028$), and FXIII (77 vs. 95%, $P = 0.0006$) than women. Women using oral contraceptives had higher activities of FVII (135 vs.109%, $P = 0.003$), FIX (110 vs. 96%, $P = 0.006$), FXI (106 vs. 95%, $P = 0.047$), and FXII (131 vs.94%, $P = 0.0003$) than women not using contraceptives. However, all these values were in reference ranges. Activities of PT (132 vs. 114%, $P = 0.0008$), FVII (123 vs. 113%, $P = 0.045$), FXI (113 vs. 96%, $P = 0.006$), and PAI-1 (32 vs. 9%, $P = 0.0004$) were increased and APTT was shorter (28 vs. 31 s, $P = 0.0004$) in smokers. Again, all these values fell to the normal range. We adjusted gender, use of contraceptives, and smoking status in all analyses.

The results of coagulation tests in the co-twins are shown in Table 1. Compared with the leaner co-twins, the heavier co-twins of the obesity discordant pairs tended to have higher levels of most coagulation factors. This difference was most striking for FIX, FXI, FXII and PAI-1 activities, and fibrinogen concentrations. In the MZ concordant group, the coagulation markers were similar in the lean and the slightly heavier co-twins.

Relationships between obesity and metabolic measures, coagulation factors, and measures of fibrinolysis

Univariate associations in individual twins. Most coagulation factors were strongly associated with BMI and other adiposity measures including the amount of subcutaneous, intra-abdominal, and liver fat. Especially, fibrinogen, PT(%), and FIX, FXI and PAI-1 activities associated strongly with the markers of obesity (Table 2). The amount of liver fat correlated

Table 2 Univariate correlations between adiposity measures, high sensitivity C-reactive protein (hsCRP), insulin sensitivity, and coagulation factors in individual MZ twins (n = 48)

	BMI	Sc fat	Ia fat	Liver fat	hsCRP[a]	M-value	Insulin
PT (%)	0.48***	0.52***	0.36**	0.17	0.60***	–0.16	0.39***
APTT (s)	–0.30	–0.18	–0.29	–0.33*	–0.01	0.40**	–0.45**
Fibrinogen (g/l)	0.60**	0.37**	0.55**	0.11	0.37**	–0.28	0.35*
FVII (%)	0.38	0.37*	0.32*	0.03	0.38*	–0.03	0.15
FVIII (%)	0.31	0.20	0.17	0.10	0.21	–0.22	0.37*
FIX (%)	0.73***	0.60***	0.60***	0.41*	0.56***	–0.54***	0.56**
FXI (%)	0.63**	0.52***	0.51***	0.31	0.47**	–0.43**	0.53***
FXII (%)	0.42*	0.32	0.50**	0.36	0.18	–0.32	0.43**
FXIII (%)	0.42*	0.25	0.39*	–0.06	0.13	0.06	0.12
VWF:RCo (%)	0.09	–0.06	0.05	–0.03	–0.02	0.02	0.23
PAI-1[b] (%)	0.73***	0.69**	0.59***	0.53**	0.62***	–0.34	0.61***
D-dimer (mg/l)	–0.11	–0.20	0.03	0.05	–0.18	0.08	0.16

Sc fat = subcutaneous fat in dm^3. Ia fat = intra-abdominal fat in dm^3.
APTT, activated partial thromboplastin time; F, coagulation factor; hsCRP, high sensitivity C-reactive protein; MZ, monozygotic; PAI-1, plasminogen activator inhibitor-1; PT, prothrombin time; VWF:RCo, von Willebrand factor ristocetin cofactor activity.
[a]n = 40 individuals. [b]n = 32 individuals.
*P < 0.05; **P < 0.01; ***P < 0.001.

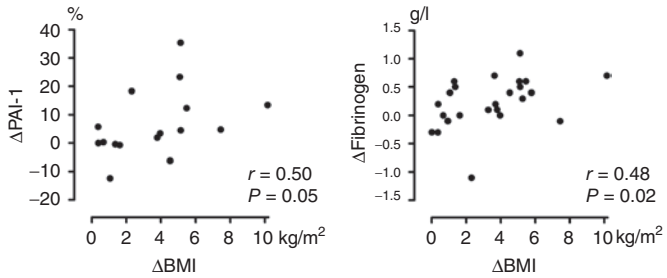

Figure 1 Associations between intrapair differences (Δ) in BMI and ΔPAI-1 (n = 16 pairs) and fibrinogen (n = 24 pairs) in monozygotic twins. P values adjusted for gender, smoking status, and use of oral contraceptives. PAI-1, plasminogen activator inhibitor-1.

positively with FIX and PAI-1 activities and negatively with APTT, whereas BMI and the amount of intra-abdominal fat correlated directly with FXII and FXIII activities.

The degree of insulin resistance and inflammation correlated with most markers of coagulation. High fasting insulin levels and low M-value, as well as elevated hsCRP, associated with hypercoagulable state (Table 2).

Univariate associations within twin pairs. In intrapair analyses within twin pairs, i.e., in analyses controlling for genetic effects within MZ twin pairs, all measures of adiposity and insulin resistance were related to fibrinogen levels and PAI-1 activity. Intrapair differences (Δ) in fibrinogen and PAI-1 activity both correlated positively with intrapair differences in BMI (Figure 1). ΔFibrinogen associated with Δsubcutaneous fat (r = 0.42, P = 0.04), ΔhsCRP (r = 0.70, P = 0.0008), and Δfasting insulin levels (r = 0.55, P = 0.005). Equally, ΔPAI-1 associated with Δsubcutaneous fat (r = 0.54, P = 0.03), Δintra-abdominal fat (r = 0.52, P = 0.04), and Δfasting insulin levels (r = 0.58, P = 0.018). Intrapair differences in FIX and FXI activities correlated with that in fasting insulin (r = 0.45, P = 0.03 and r = 0.40, P = 0.05 respectively). ΔD-dimer correlated negatively with Δinsulin sensitivity (r = –0.43, P = 0.04).

Multivariate associations within MZ twin pairs. Multivariate analyses were performed using intrapair differences in MZ pairs to assess which of the previously mentioned covariates independently explained the perturbed coagulation and fibrinolytic variables of D-dimer and PAI-1 in obese co-twins. We entered differences in body fat distribution (Δsubcutaneous fat, Δintra-abdominal fat, and Δliver fat), Δfasting insulin, ΔhsCRP, gender, smoking status, and use of oral contraceptives as independent variables in the models. ΔFibrinogen was independently related to ΔhsCRP (β = 0.35 ± 0.15, P = 0.04; adjusted whole model R^2 = 0.29, P = 0.18). ΔFXII was independently influenced by use of oral contraceptives (β = 24.5 ± 9.9, P = 0.04; adjusted whole model R^2 = 0.59, P = 0.03). Within pairs, no other measures of adiposity or metabolism were related to the coagulation or fibrinolysis markers in the multivariate analyses.

Within twin-pair similarity of measures of coagulation and fibrinolysis. Finally, we calculated within-pair intraclass correlations in order to quantify the degree to which twins resembled each other in their measures of coagulation and fibrinolysis. FVII, FVIII, FXI, FXII, FXIII, and VWF activities, fibrinogen, PT, and APTT all appeared to be under strong familial influence in both concordant (r = 0.86–0.97, P < 0.003) and discordant (r = 0.73–0.92, P < 0.03) MZ twin pairs. Yet, FXI, FXII and PAI-1 activities, and fibrinogen concentrations were significantly increased in the obese MZ co-twins (Table 1). The intraclass correlation for FIX activity was strong in weight-concordant MZ twins (r = 0.95, P = 0.001) but weaker in MZ discordant twins (r = 0.63, P = 0.14).

DISCUSSION

In this study, we analyzed how obesity influences variables of blood coagulation and fibrinolysis in a unique genetically controlled sample of obesity-discordant MZ twin pairs. Their obesity was of a recent nature as the obese co-twins aged 23–27 at the time of the study had become obese only after puberty (18). We demonstrated a consistent increase in several markers of coagulation and fibrinolysis in obesity in these healthy young adults. The activities of FIX, FXI, and FXII, fibrinogen and PAI-1 were enhanced most in obese co-twins.

In addition to these influences of acquired obesity, we found that most of the coagulation factor activities (FVII, FVIII, FIX, FXII, FXIII, VWF, PT, and APTT) and fibrinogen were extremely similar especially within weight-concordant MZ co-twins, suggesting a tight genetic regulation of the individual coagulation factors. Thus, two main observations on the obesity-related prothrombotic state can be made in our study: (i) it arises early in still otherwise healthy young adults, soon after the development of obesity, and (ii) its origin is a complex mixture of each individual genetic background and lifestyle.

Controlling for genetic background, within-pair differences in fibrinogen and PAI-1, the two crossroad factors of blood coagulation and regulation of fibrinolysis, related to the differences in measures of obesity (BMI, subcutaneous, and intra-abdominal fat). Previous studies have shown that increased PAI-1 activity is compatible with widely documented obesity-linked derangement of fibrinolysis (4,5). Elevated fibrinogen levels predicted with future cardiovascular events in a large meta-analysis (28,29). In return, weight loss and exercise have been documented to decrease the activity of PAI-1 (9). Again, decrease in fibrinogen levels occurred after weight loss upon bariatric surgery in morbidly obese patients (30).

The activity of PAI-1 showed wide variation. We took this in consideration by log-transformation since the data was non-normally distributed. The highest PAI-1 levels were observed in smokers. As there were four current smokers in the group of weight-discordant twins (two in the leaner and two in the heavier co-twins) and none in the group of weight-concordant pairs, smoking may explain part of the increased PAI-1 levels in the weight-discordant pairs. Thus, the effect of smoking was adjusted in our analyses. However, smoking did not fully explain why the discordant pairs would have higher PAI-1 levels than the concordant pairs. Without the smokers, leaner vs. heavier PAI-1 levels were 8.0% vs. 20.4% in the discordant and 3.9% vs. 3.9 % in the concordant pairs.

Increased FVII, FVIII, and VWF levels are associated with obesity, the presence of premature atherosclerotic disease *and insulin resistance* (31–33). *However,* obesity did not associate with increased FVIII or VWF activity in our study. The discrepancy with some earlier observations that obesity generally raises levels of VWF (4) may arise from the young age of our subjects. Increased VWF refers to endothelial dysfunction (4) which may not occur in young individuals having yet fairly modest metabolic sequelae of obesity.

Elevated FIX, FXI, FXII, and FXIII are all risk markers for thrombosis (34–37) and this study revealed how obesity modulates their activities. Earlier evidence of increased activities of these clotting factors in obesity originates from studies of closely related metabolic disorders. For example, FIX is elevated in type 2 diabetes and in conditions with increased circulating interleukin-6 concentrations (38–40). FXII has been shown to correlate with BMI (41), compatible with our results. The FXIII gene was identified as an obesity gene in a genome-wide association study by Naukkarinen *et al.* (42), constituting a new link between obesity and increased risk of thrombosis.

In our study, obese co-twins displayed lower insulin sensitivity and hyperinsulinemia compared with their leaner counterparts, independent of the genetic background. In individual twins, fasting insulin concentrations correlated positively with fibrinogen, PT, FVIII, FIX, FXI, FXII, and PAI-1 and negatively with APTT. FIX, FXI, and D-dimer were associated with the insulin resistant state independent of genetic factors within pairs. Therefore our results imply that the prothrombotic and hypofibrinolytic *milieu* in acquired obesity concurs with insulin resistance. Of note, phenotypic correlations between insulin resistance and hemostatic factors have been reported in earlier twin studies (32) and their coexistence may also arise from pleiotropically acting genes (32).

hsCRP correlated with fibrinogen, PT, FVII, FIX, FXI, and PAI-1 activities (**Table 2**). In this study, the heavier MZ co-twins had higher hsCRP concentrations than their leaner counterparts and this difference independently predicted variation of fibrinogen in multivariate analysis in twin pairs. These results are in line with earlier reports on the associations between a prothrombotic state and inflammation (6,38,43,44). Sakkinen *et al.* suggested that fibrinogen to a larger extent reflects inflammation rather than procoagulation in obesity (45). Inflammatory cytokines, such as interleukin-1, interleukin-6, and tumor necrosis factor-α, produced by adipose tissue stimulate hepatocytes to produce PAI-1 and acute phase reactants, some of which are procoagulants (43,44,46). The extent of inflammation associates with adipose tissue mass and decreases upon weight loss (47).

We observed a particularly strong familial link for the levels of most coagulation factors. Intraclass correlation analyses showed high-degree of resemblance between the MZ co-twins despite of acquired weight differences with ensuing differences in the levels of coagulation factors. Interestingly, however, the within-pair correlation of FIX weakened with weight-discordance, suggesting that obesity modifies the pathways responsible for the production of FIX. Significant genetic influences on fibrinogen, FVII, and VWF have been reported in earlier twin studies (11,48,49). A similar genetic resemblance between MZ twins was reported for FVIII, FXIII, and PAI-1 (11). In our study, intraclass correlation point estimates were higher than in these other studies including older subjects. This implies that the relative contribution of environmental influences over genes is likely to increase with age.

Adiposity may functionally alter the function of several genes in the coagulation cascade. One such gene is hepatocyte nuclear factor-4α, a transcription factor which regulates the synthesis of FVII, FIX, FXI, FXII, and FXIIIB in liver (50,51). The activities of some of these coagulation factors (FIX, FXI, FXII) were

elevated in the obese co-twins and correlated with adiposity markers in our study. Another regulator of coagulation and fibrinolysis is CCAAT/enhancing binding protein-α, a transcription factor protein expressed both in liver and in adipose tissue (52). Enhanced expression of CCAAT/enhancing binding protein-α augments the levels of FIX (52), and its binding site is also present in the PAI-1 gene (53). Accordingly, both FIX and PAI-1 activities were increased in obese co-twins and correlated strongly with intra-abdominal and subcutaneous fat in our data. Finally, the observed prothrombotic state observed in our study may also arise from disturbed clearance of coagulation factors in liver. The low-density lipoprotein receptor-related protein-1 is expressed in hepatocytes and mediates intracellular degradation of several molecules including FVIII, FIX, D-dimer, and PAI-1 (54,55).

Weight—discordance in monozygotic twins is extremely rare and we were only able to find 14 discordant pairs for this study. The small number of study subjects may set limitations in the interpretation of the results of our study but our results are in line with earlier reports showing that obesity is a procoagulant state. We showed that the measures of coagulation and fibrinolysis are extremely similar in MZ co-twins, implying that genetic factors may confound studies comparing obese and nonobese subjects from studies of individuals. Therefore, it is an important advantage that we here were able to control for the genetic effects by using the MZ obesity-discordant design and showed that despite significant heritable components, acquired obesity impairs most coagulation factors. As a limitation, we would also like to note that several markers of fibrinolysis–tissue plasminogen activator, urokinase-type plasminogen activator, plasminogen, and antiplasmin–measuring the global fibrinolytic activity were not included in our analyses. This study demonstrated that derangements of coagulation and fibrinolysis are present already in early adulthood in obese but otherwise healthy subjects. The levels of coagulation factors are under strong genetic control, but obesity in parallel with insulin resistance and inflammation influence the activity of most measures of coagulation and fibrinolysis in a complex manner requiring further mechanistic studies.

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DISCLOSURE

The authors declared no conflict of interest.

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Original Article
OBESITY BIOLOGY AND INTEGRATED PHYSIOLOGY

Obesity

Acquired Liver Fat Is A Key Determinant of Serum Lipid Alterations in Healthy Monozygotic Twins

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Objective: The effects of acquired obesity on lipid profile and lipoprotein composition in rare BMI-discordant monozygotic (MZ) twin pairs were studied.

Design and Methods: Abdominal fat distribution, liver fat (magnetic resonance imaging and spectroscopy), fasting serum lipid profile (ultracentrifugation, gradient gel-electrophoresis, and colorimetric enzymatic methods), and lifestyle factors (questionnaires and diaries) were assessed in 15 BMI-discordant (within-pair difference [Δ] in BMI >3 kg/m^2) and nin concordant (ΔBMI <3 kg/m^2) MZ twin pairs, identified from two nationwide cohorts of Finnish twins.

Results: Despite a strong similarity of MZ twins in lipid parameters (intra-class correlations 0.42-0.90, $P < 0.05$), concentrations of apolipoprotein B (ApoB), intermediate-density lipoprotein cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein 3a% (HDL3a%), and HDL3c% were higher ($P < 0.05$) and those of HDL cholesterol, HDL2-C, and HDL2b% were lower ($P < 0.01$) in the heavier co-twins of BMI-discordant pairs. The composition of lipoprotein particles was similar in the co-twins. When BMI-discordant pairs were further divided into liver fat-discordant and concordant (based on median for Δliver fat, 2.6%), the adverse lipid profile was only seen in those heavy co-twins who also had high liver fat. Conversely, BMI-discordant pairs concordant for liver fat did not differ significantly in lipid parameters. In multivariate analyses controlling for Δsubcutaneous, Δintra-abdominal fat, sex, Δsmoking and Δphysical activity, Δliver fat was the only independent variable explaining the variation in ΔApoB, Δtotal cholesterol, and ΔLDL-C concentration.

Conclusions: Several pro-atherogenic changes in the amounts of lipids but not in the composition of lipoprotein particles were observed in acquired obesity. In particular, accumulation of liver fat was associated with lipid disturbances, independent of genetic effects.

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Introduction

Obesity-associated derangements of plasma lipid profile are a widely documented risk factor for cardiovascular disease (1). Body fat depots differ in their contribution to lipid metabolism. Intra-abdominal (ia) fat rather than subcutaneous (sc) or gynoid body fat is associated with an atherogenic lipid profile and increased cardiovascular risk (2,3). Ectopic fat in liver contributes significantly both to serum triglyceride (TG) and high-density lipoprotein cholesterol (HDL-C) levels (4). The derangement of lipoprotein composition due to liver steatosis is already seen in children presenting higher amounts of large very low-density lipoprotein (VLDL), small dense low-density lipoprotein (LDL), and smaller HDL particles (5). Characterizing the individuals at highest cardiovascular risk is a challenge. Not all obese individuals present the same harmful metabolic sequelae probably due to a different predisposition to accumulation of ectopic fat

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(6,7). A subgroup of obese individuals remains insulin sensitive and presents with normal TG and HDL-C concentrations (8). Detailed lipid concentrations and compositions and their relationships to body fat distribution in obesity are poorly characterized.

Derangements in the lipid profile are determined by complex combinations of genetic and lifestyle factors (9). In our previous studies, we provided evidence for large genetic influences on variations in LDL-C (79%), HDL-C (73%), and TGs (64%), as has been previously reported elsewhere (10). In large genome-wide association studies, several single nucleotide polymorphisms associate with lipid levels in humans (11). Moderate heritability estimates were observed for LDL peak particle size (49%) and HDL mean particle size (56%) and HDL subspecies (46-63%) (10), confirming that substantial environmental influences also exist. The sources of those environmental influences are variable and may be difficult to distinguish from genetic and environmental influences. Obesity and regional fat distribution have a substantial heritability (12) and many genes underlying obesity have been identified (13). BMI-discordant monozygotic (MZ) twins offer a unique model where the influence of acquired obesity on lipid profile can be disentangled from genetic factors, as in this design the lean and heavy subjects are completely matched for any variations in the genetic background (DNA sequence) (14).

We aimed to determine the associations between acquired obesity and serum lipid profile with a special emphasis on fat distribution, sc, ia, and liver fat in rare MZ twin pairs discordant for BMI. In addition, we tested the similarity of lipoprotein particle quantity and quality in MZ twins, to evaluate the genetic similarity within the twin pairs. We also aimed to carefully adjust the confounding effects of lifestyle factors on lipid profile in our analyses.

Methods

Subjects

The twins included in this study were recruited from two population-based, longitudinal studies, FinnTwin16 (FT16) and FinnTwin12 (FT12), each consisting of five consecutive birth cohorts of Finnish twins (15). In FT16, twins born between 1975 and 1979 were followed up by questionnaires at 16, 17, 18.5, and 22-27 years of age (response rates 83–97%, $n = 5{,}601$ at baseline). In FT12, twins born between 1983 and 1987 were sent questionnaires at 11-12, 14, and 17.5 years (response rates 74-92%, $n = 5{,}184$ at baseline). This study included an intensive subsample from the last follow-ups of both FT12 and FT16 studies including 15 pairs (6 male and 9 female pairs) discordant for BMI (within-pair difference in BMI $\geq$3 kg/m^2 and 10-30 kg in weight) and 9 pairs (5 male and 4 female pairs) concordant for BMI (within-pair difference in BMI $<$3 kg/m^2). Within-pair difference of $\geq$3 kg/m^2 represents the top 5% most discordant pairs in the cohorts. All pairs were of Caucasian origin with a mean age of 28.1 years (range 22.8-33.1 years). Except for one obese co-twin who had recently developed type 2 diabetes and was on insulin therapy and another obese co-twin having an inactive ulcerative colitis and being on mesalazine and azathioprine treatment, the subjects were healthy and did not take any medications. The twins' weights had been stable for at least 3 months prior to the study. None of the female subjects were pregnant or lactating. One female subject used oral contraceptives. Monozygosity was confirmed by genotyping of 10 informative genetic markers (16). The study protocols were approved the ethical committee the Hospital District of Helsinki and Uusimaa, Finland. Written informed consent was obtained from all participants.

Diet, smoking, and physical activity

Energy and macronutrient intakes were assessed from 3-day food records and analyzed by Diet32, which is based on a national Finnish database for food composition (17). Habitual alcohol intake was assessed by a structured questionnaire. The non-smokers comprised never smokers, former and occasional smokers, and the current smokers comprised those who were daily smokers ($n = 6$ individuals). Physical activity was assessed using the Baecke physical activity questionnaire, which derives an index for physical activity in total (total index) and separate indices for physical activity at work (work index), sports activities during leisure time (sport index), and physical activity during leisure time excluding sports (leisure time index) (18).

Body composition

Weight and height were measured after 12-h overnight fast to calculate BMI. Body composition was measured using whole body dual X-ray absorptiometry (DXA) scans (software version 8.8, Lunar Prodigy, Madison, WI,USA). A standardized procedure at least 4 h after a light meal with empty bladder was used to avoid differences in the hydration status. Whole body fat percentage was calculated as fat mass/(fat mass + lean mass + bone mineral content).

Magnetic resonance experiments

The magnetic resonance (MR) measurements were performed on a clinical 1.5 T imager (Avanto, Siemens, Germany, Erlangen). To allow measurement of abdominal fat distribution, a T1-weighted axial image stack of 16 slices with thickness of 10 mm and gap of 0 mm was centered at L4/L5 intervertebral disk. Selective fat excitation was used to obtain images with a standard body coil. MR images were analyzed using SliceOmatic v4.3 segmentation software (Tomovision, Montreal, Canada). The areas of sc and ia fat tissue were measured for each slice using a region-growing routine. The results were expressed as total volumes of sc fat and ia fat.

For liver-fat analyses, point resolved spectroscopy (PRESS) localization technique with repetition time (TR)/echo time (TE) of 3,000/30 ms and 16 acquisitions was used to obtain non-suppressed liver spectra. Orthogonal three plane images were used for localization of the cubic 8-27 cm^3 voxel of interest within the right lobe of the liver avoiding signal contamination from vascular structures, gallbladder, and adipose tissue. The MRS data were collected using a flex surface coil in combination with spine coils. The liver spectra were analyzed with jMRUI v3.0 software (19) using the AMARES algorithm (20). Areas of water signal at 4.7 parts per million (ppm) and methylene signal from intracellular TGs at 1.3 ppm were determined using a line-fitting procedure. Spectroscopic intracellular TG content was expressed as methylene/(water + methylene) signal area $\times$ 100 and the values were further converted to mass fractions as described earlier (21).

Biochemical analyses

Venous blood samples from the study subjects were drawn after an overnight fast and serum and EDTA plasma were separated by centrifugation and stored at -80°C until analysis for lipid profile. Plasma concentrations of apolipoprotein A1 (ApoA1) and apolipoprotein B (ApoB) were measured by immunoturbidometric methods (for ApoA1, Wako Chemicals GmbH and for ApoB, Orion Diagnostica, Espoo, Finland). Serum apolipoprotein C3 (ApoC3)

concentration was quantitated by ELISA (22). Serum total cholesterol (TC) and TGs were determined using an automated Konelab 60i analyzer (Thermo Fisher Scientific Oy) by enzymatic methods (Refs. 981812 and 981301).

Fasting serum lipoproteins (VLDL, intermediate-density lipoprotein [IDL], LDL, HDL2, and HDL3) were separated by sequential flotation ultracentrifugation using a modification of the method of Havel et al. (23). Lipoprotein compositions were analyzed using enzymatic methods by Konelab 60i. HDL was separated and isolated by ultracentrifugation from 0.5 ml plasma (24). Distribution of HDL2b, 2a, 3a, 3b, and 3c subspecies and HDL mean particle size were determined by native gradient gel electrophoresis as previously described with minor modifications (24,25). The molecular size intervals for HDL subspecies 2b, 2a, 3a, 3b, and 3c were used, and for each subspecies, the relative area under the densitometric scan was reported. Mean HDL particle size was calculated by multiplying the mean size of each HDL subclass by its relative area under the densitometric scan. LDL peak particle diameters determined using 1 mm 2-10% linear non-denaturating polyacrylamide gradient gels (26).

Statistical methods

Statistical analyses were performed with Stata statistical software (release 11.0; Stata Corporation, College Station, TX, USA). Results are expressed as mean ± SE unless otherwise specified. Twin similarity was assessed using sex-adjusted intra-class correlations (ICCs). Mean values of leaner and heavier co-twins are shown unadjusted. Wilcoxon matched-pairs signed-ranks test was performed to test whether heavier and leaner co-twins differed significantly from each other. Within-pair differences (Δ) were calculated by subtracting the leaner co-twin's value from the heavier co-twin's value and using all twin pairs in analyses. Mann–Whitney *U*-test was used to test whether Δlipids differed in discordant vs. concordant groups. Pearson partial correlations adjusted for sex were used to calculate correlations between Δs of different adiposity measures. The effects of acquired adiposity on serum lipid profiles were calculated using Pearson partial correlations (Δeach adiposity measure vs. Δeach lipid value) adjusted for sex, Δphysical activity (total index), and Δsmoking status. Similarly, the effects of Δphysical activity on Δlipids were tested by Pearson correlation adjusted for sex and Δsmoking. Multiple regression analyses included Δeach lipid value against Δsc fat, Δia fat and Δliver fat, sex, Δphysical activity, and Δsmoking in the same model. Because further adjustment for Δpercentage of energy from macronutrients or alcohol did not substantially change these results, they were not included in the final models.

Results

Physical characteristics

Physical characteristics and the lipid profiles of MZ twin pairs are summarized in Table 1. Briefly, within-pair differences (Δ) in BMI ranged from 3.1 to 9.4 kg/m^2 in discordant pairs and from 0.1 to 2.3 kg/m^2 in concordant pairs. The heavier co-twins of BMI-discordant pairs had on average 1.6 times the amount of sc fat, 2.0 times that of ia fat, and 5.1 times the amount of liver fat than their leaner counterparts. These parameters did not differ between the heavier and leaner co-twins in BMI-concordant twin pairs. To further analyze the influence of liver fat we divided BMI-discordant twin pairs into two subgroups based on median for Δliver fat (2.6%) in this study sample: seven BMI-discordant pairs (Δweight 16 kg) where both co-twins had low liver fat percentages (from 0.1 to 1.6%) had no differences in liver fat (Δliver fat 0-0.2%, $P = 0.40$). The remaining eight BMI-discordant pairs (Δweight 17 kg) differed significantly for liver fat: the heavier co-twins' liver fat% ranged from 3.8% to 9.4% (Δliver fat 2.6-9.0%, $P = 0.012$).

Within-pair similarity of lipid profile

At first, sex-adjusted ICC was determined from all twins to assess familial, probable genetic control over lipoprotein composition. In pairs concordant for BMI, ICC coefficients ranged from 0.51-0.93 (all $P < 0.05$) for concentrations of lipids, phospholipids, and proteins in VLDL, LDL, IDL, and HDL particles as well as lipoprotein particle masses. Equally, ICC analysis revealed high within-pair similarity in apolipoproteins ApoB and ApoC3 (0.70 and 0.81 respectively, both $P < 0.05$), TC concentration (0.85, $P = 0.0004$), all HDL subclasses (0.60-0.77, all $P < 0.05$), HDL mean particle size (0.80, $P = 0.01$), and marginal significance in ApoA1 (0.56, $P = 0.06$).

Within-pair similarity was significant even in BMI-discordant twins for concentrations of lipids, phospholipids, and proteins in IDL and HDL2 particles (0.48-0.64, $P < 0.05$). However, we identified some notable exceptions. In VLDL, within-pair similarity in particle composition weakened with weight-discordance as ICC coefficient range 0.11-0.35 was much lower and statistically non-significant compared with the marked resemblance in concordant pairs (0.70-0.85, $P < 0.01$). There was very little within-pair resemblance for ApoB, VLDL-C, LDL-C, HDL2a%, total TG concentration, and VLDL and LDL masses in BMI-discordant twin pairs (0.12-0.38, $P = 0.07$-0.29). There was no within-pair similarity in LDL peak particle size and only modest similarity in HDL3 particle in this data.

Differences in heavier vs. leaner co-twins in lipid profile and lipoprotein composition

The BMI-concordant MZ co-twins did not differ for their lipid profile (Table 1). In BMI-discordant pairs, the heavier co-twins had significantly higher levels of ApoB ($P = 0.01$), TC ($P = 0.05$), LDL-C ($P = 0.05$), IDL-C ($P = 0.01$), and HDL3c% ($P = 0.003$), as well as higher IDL and LDL masses ($P = 0.02$ and 0.05 respectively). Levels of HDL-C, HDL2-C, and HDL2b% were lower ($P = 0.01$ for all) and HDL mean particle size was smaller ($P = 0.01$) in the heavier co-twins. There were no differences in concentrations of ApoA1, ApoC3, VLDL-C and HDL3-C, LDL peak particle size, VLDL, or HDL masses between the co-twins. Neither total TG (Table 1) nor VLDL-TG (54.4 ± 5.7 mg/dl vs. 69.2 ± 7.7, $P = 0.15$) concentration differed between leaner and heavier co-twins.

Acquired obesity associated with only minor changes in the lipoprotein particle composition when assessing qualitative composition of each lipoprotein particle (data not shown). The heavier co-twins of the discordant pairs had significantly higher percentages of IDL free cholesterol (7.7 vs. 7.0%, $P = 0.05$) and cholesterol esters (10.6 vs. 9.0%, $P = 0.05$) in relation to other components within IDL particle when compared with their leaner counterparts. HDL2 particles were enriched with TG's in the heavier co-twins (6.1 vs. 5.1%, $P = 0.02$).

Correlations between adiposity, physical activity, and lipid profile within twin pairs

In analyses controlling for genetic effects within all MZ twin pairs, Pearson partial correlation coefficients between Δmarkers of

TABLE 1 Physical characteristics and lipid profiles of monozygotic twin pairs

	BMI-discordant $n = 15$ pairs		BMI-concordant $n = 9$ pairs	
	ΔBMI >3 kg/m^2		ΔBMI <3 kg/m^2	
	Leaner	Heavier	Leaner	Heavier
Adiposity measures				
BMI (kg/m^2)	24.8 ± 1.0	30.3 ± 1.0***	27.6 ± 1.1	29.3 ± 1.1**
Body fat %	32.2 ± 2.4	40.6 ± 2.0***	31.6 ± 3.1	33.0 ± 2.6
Sc fat (dm^3)	3.4 ± 0.4	5.4 ± 0.1***	3.6 ± 1.0	3.9 ± 0.5
Ia fat (dm^3)	0.6 (0.3–0.8)	1.1 (0.7–1.5)***	1.1 (0.6–1.5)	1.1 (0.6–1.4)
Liver fat (%)	0.6 (0.4–1.1)	3.8 (0.6–6.1)**	1.3 (0.5–1.6)	1.0 (0.5–1.7)
Apolipoproteins				
ApoA1 (mg/dl)	128.9 (119.3–172.5)	129.9 (113.0–144.8)	126.4 (112.6–130.5)	127.6 (118.8–142.6)
ApoB (mg/dl)	69.8 ± 3.3	81.1 ± 4.0**	81.0 ± 9.6	81.1 ± 7.6
ApoC3 (mg/dl)	9.5 ± 0.7	9.4 ± 0.7	9.4 ± 2.0	9.7 ± 1.3
Serum TGs and cholesterol				
TG (mg/dl)	93.8 (74.3–115.1)	112.4 (87.6–130.1)	83.2 (63.7–201.8)	98.2 (74.3–104.4)
TC (mg/dl)	174.6 ± 5.5	183.7 ± 6.2*	173.4 ± 14.5	176.6 ± 13.4
VLDL-C (mg/dl)	9.4 ± 1.0	12.4 ± 1.8	14.1 ± 4.9	11.3 ± 3.2
LDL-C (mg/dl)	98.4 ± 5.8	110.4 ± 5.6*	104.1 ± 11.1	108.4 ± 11.5
IDL-C (mg/dl)[a]	3.8 (2.9–4.3)	5.0 (3.1–6.7)**	3.7(2.5–5.5)	2.6 (2.3–5.4)
HDL-C (mg/dl)[a]	62.7 ± 3.7	51.2(46.0–61.8)**	50.9 ± 3.6	52.8 ± 3.8
HDL2-C (mg/dl)	31.9 ± 3.5	21.3 (15.4–25.0)**	21.1 ± 3.9	15.3 (14.8–21.5)
HDL3-C (mg/dl)	30.8 ± 1.2	31.4 ± 1.2	29.7 ± 1.8	32.4 ± 1.8
Lipoprotein particle mass and size				
VLDL mass (mg/dl)	95.2 ± 8.8	121.6 ± 14.0	143.2 ± 44.3	123.8 ± 33.1
LDL mass (mg/dl)	243.6 ± 11.4	254.3 (228.0–316.8)*	267.7 ± 24.6	276.7 ± 26.7
IDL mass (mg/dl)	25.3 ± 2.3	29.0 ± 2.9*	25.3 ± 3.7	24.8 ± 3.1
HDL mass (mg/dl)	393.4 ± 23.1	362.5 ± 22.1	334.5 ± 15.4	351.8 ± 25.6
LDL size (nm)	26.6 ± 0.2	26.3 ± 0.2	26.1 ± 0.4	25.9 ± 0.3
HDL size (nm)	9.6 ± 0.1	9.4 ± 0.1**	9.2 ± 0.2	9.1 ± 0.1
HDL subclasses				
HDL2a%	27.0 ± 1.0	27.2 ± 0.8	24.4 ± 1.9	25.8 ± 1.4
HDL2b%	36.0 ± 2.9	28.7 ± 3.0**	23.7 ± 5.0	20.3 ± 3.1
HDL3a%	24.3 ± 1.6	27.5 ± 2.1	30.5 ± 2.1	33.7 ± 1.9
HDL3b%	9.2 ± 1.1	11.2 ± 1.1	15.7 ± 3.1	15.0 ± 1.9
HDL3c%	3.5 ± 0.6	5.4 ± 1.1**	5.7 ± 1.5	5.2 ± 0.8

Data are mean ± SE, non-normally distributed data are median (25–75% inter-quartile range). Wilcoxon signed ranks test leaner vs. heavier co-twin, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; ApoC3, apolipoprotein C3; HDL-C, high density lipoprotein cholesterol; ia, intra-abdominal; IDL-C, intermediate density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; sc, subcutaneous; TC, total cholesterol; TG, triglycerides; VLDL-C, very low density lipoprotein cholesterol.

[a]The within-pair difference significantly different ($P < 0.05$) in discordant vs. in concordant pairs (Mann-Whitney U-test).

adiposity, and Δlipid profile adjusted for sex, Δphysical activity, and Δsmoking are presented in Table 2. ΔBMI, Δia, Δsc, and Δliver fat were strong correlates of atherogenic lipid parameters independent of genetic effects. After further adjusting for Δliver fat, correlations became weaker for many variables.

In contrast, Δphysical activity adjusted for sex, Δsmoking, and ΔBMI associated strongly with a favorable lipid profile: ApoB: $r = -0.53$, LDL-C: $r = -0.54$, HDL-C: $r = 0.47$, LDL mass: $r = -0.48$, HDL mass: $r = 0.46$, HDL3b: $r = -0.45$ ($P < 0.05$ for all).

Most adiposity measures were significantly correlated with each other. Sex-adjusted partial correlations were as follows: ΔBMI and Δsc fat: $r = 0.93$, $P < 0.001$, ΔBMI and Δia fat: $r = 0.72$, $P < 0.001$, ΔBMI and Δliver fat: $r = 0.35$, $P = 0.09$, Δia fat and Δsc fat: $r = 0.62$, $P = 0.002$, Δia fat and Δliver fat: $r = 0.52$, $P = 0.01$.

Because of the high correlation between the measures of adiposity, multivariate regression analyses were performed to assess which component of body fat distribution independently explained pro-atherogenic changes in lipid profile. We entered within-pair differences in body fat distribution (Δsc fat, Δia fat, and Δliver fat), sex,

TABLE 2 Pearson partial correlations between within-pair differences (Δ) in adiposity and Δlipid parameters, adjusted for sex, Δsmoking, and Δphysical activity in monozygotic twin pairs (n = 24 pairs)

	ΔLiver fat%	ΔBMI	ΔBMI adjusted for ΔLiver fat	ΔSc fat	ΔSc fat adjusted for ΔLiver fat	ΔIa fat	ΔIa fat adjusted for ΔLiver fat
Apolipoproteins							
ΔApoA1	0.06	−0.09	−0.12	−0.18	−0.20	−0.10	−0.14
ΔApoB	0.56**	0.55*	0.45*	0.44*	0.39	0.49*	0.32
ΔApoC3	0.09	0.00	−0.03	−0.15	−0.18	0.06	0.02
Serum TGs and cholesterol							
ΔTG	0.47*	0.52*	0.43(*)	0.38	0.32	0.57**	0.46*
ΔTC	0.53*	0.31	0.17	0.30	0.21	0.23	0.00
ΔVLDL-C	0.31	0.56*	0.50	0.44*	0.39	0.60**	0.54*
ΔLDL-C	0.40(*)	0.21	0.08	0.24	0.17	0.12	−0.06
ΔIDL-C	0.40(*)	0.53*	0.46*	0.44*	0.39	0.45*	0.33
ΔHDL-C	−0.12	−0.38	−0.37	−0.37	−0.36	−0.39(*)	−0.38
ΔHDL2-C	−0.32*	−0.37	−0.29	−0.32	−0.27	−0.41(*)	−0.32
ΔHDL3-C	0.42(*)	−0.14	−0.33	−0.22	−0.36	−0.07	−0.31
Lipoprotein mass and size							
ΔVLDLmass	0.43(*)	0.52*	0.44(*)	0.38	0.32	0.56**	0.45*
ΔIDLmass	0.11	0.21	0.18	0.17	0.15	0.13	0.09
ΔLDLmass	0.44*	0.35	0.23	0.36	0.29	0.27	0.09
ΔHDLmass	0.07	−0.10	−0.13	−0.16	−0.19	−0.08	−0.13
ΔLDL size	−0.08	−0.32	−0.31	−0.28	−0.27	−0.43(*)	−0.44(*)
ΔHDL size	−0.30	−0.48*	−0.42(*)	−0.47*	−0.43(*)	−0.41(*)	−0.33
HDL subclasses							
ΔHDL2a%	−0.06	−0.05	−0.03	−0.09	−0.07	−0.02	0.06
ΔHDL2b%	−0.30	−0.46*	−0.40(*)	−0.45*	−0.41(*)	−0.42(*)	−0.34
ΔHDL3a%	0.21	0.31	0.26	0.32	0.28	0.24	0.17
ΔHDL3b%	0.31	0.44*	0.37	0.43(*)	0.38	0.30	0.19
ΔHDL3c%	0.09	0.18	0.16	0.18	0.17	0.27	0.25

(*)$P < 0.08$, *$P < 0.05$, **$P < 0.01$.
Sc fat, subcutaneous fat in dm^3; Ia fat, intra-abdominal fat in dm^3.

Δsmoking status, and Δphysical activity as independent variables in the models. Out of all body fat depots, Δliver fat was the only one independently explaining the variation in ΔApoB ($\beta = 2.2 \pm 1.0$, $P = 0.05$; whole model adjusted $R^2 = 0.45$, $P = 0.01$), ΔTC ($\beta = 3.6 \pm 1.4$, $P = 0.02$; $R^2 = 0.37$, $P = 0.04$), ΔLDL-C ($\beta = 2.9 \pm 1.3$, $P = 0.04$; $R^2 = 0.56$, $P = 0.003$), ΔHDL3-C ($\beta = 0.7 \pm 0.3$, $P = 0.04$; $R^2 = 0.08$, $P = 0.32$), and also marginally ΔLDL mass ($\beta = 5.7 \pm 2.9$, $P = 0.07$; $R^2 = 0.48$, $P = 0.01$). ΔPhysical activity remained significant in models explaining ΔLDL-C ($\beta = -10.4 \pm 2.8$, $P = 0.002$; $R^2 = 0.56$, $P = 0.003$), ΔLDL mass ($\beta = -16.7 \pm 6.2$, $P = 0.016$; $R^2 = 0.48$, $P = 0.01$), and marginally ΔHDL3b ($\beta = -1.5 \pm 0.7$, $P = 0.06$; $R^2 = 0.28$, $P = 0.088$).

Influence of liver fat on lipid profile in twins from BMI-discordant pairs

Division of the BMI-discordant pairs based on their within-pair differences for liver fat revealed an interesting splitting of the results. Despite the marked difference in weight in both groups, only those obese co-twins who also had high liver fat, showed a pro-atherogenic lipid profile (Figure 1). In this group, the heavier co-twins had higher levels of ApoB, TC, LDL-C, IDL-C, LDL mass, HDL3c and lower levels of HDL-C, smaller HDL mean particle size ($P < 0.05$), and marginally higher TG concentrations ($P = 0.07$) than their leaner counterparts. ApoA1, ApoC3, VLDL-C, VLDL, IDL and HDL masses, LDL peak particle size, or other HDL subclasses did not differ between the co-twins neither in liver fat discordant nor in liver fat concordant pairs (data not shown). In BMI-discordant but liver fat concordant pairs, the lipid profiles were very similar between the co-twins: the only difference noted was a slightly smaller HDL peak particle size (9.7 vs. 9.5 nm, $P = 0.05$) in the heavier co-twins.

Discussion

In this study, we were able to demonstrate that acquired obesity is associated with several pro-atherogenic derangements in the lipoprotein metabolism especially when obesity is accompanied with a fatty liver. In our study, we used a unique sample of MZ twin pairs discordant for BMI, which allowed us to estimate the effects of acquired adiposity in heavier and leaner individuals matched for genetic background. A further advantage of this study was that half

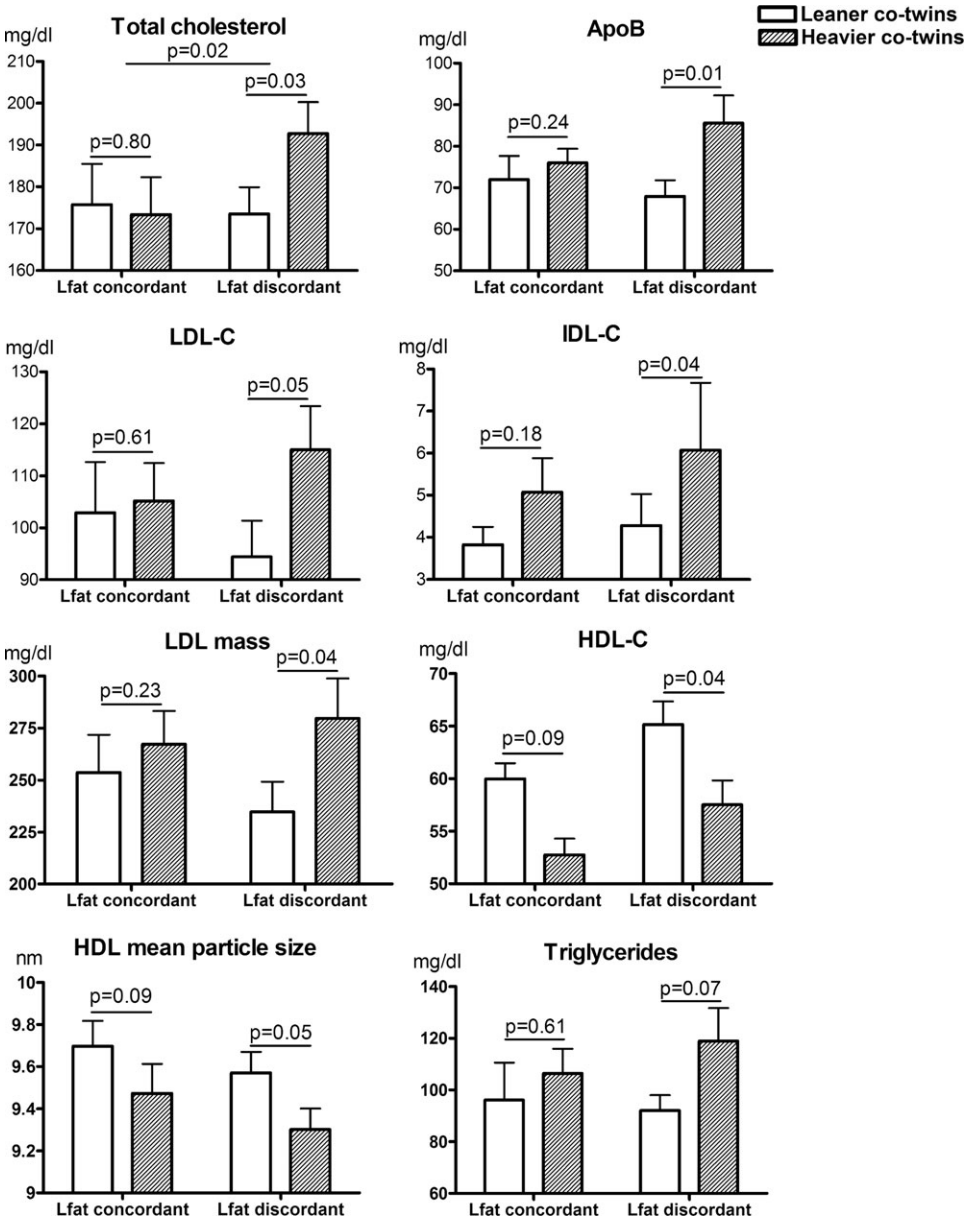

FIGURE 1 Lipid profiles in BMI-discordant twins divided into two groups based on the median of within-pair difference (Δ) in liver fat 2.6%: (1) concordant (Δlfat <2.6%) or (2) discordant (Δlfat ≥2.6%) for liver fat. Both groups were equally discordant for overall obesity (mean Δbody weight 16 and 17 kg). Data are mean ± SE. *P* values: leaner vs. heavier co-twins in Wilcoxon signed ranks tests and liver fat discordant vs. concordant pairs in Mann–Whitney *U*-test. Liver fat concordant group, $n = 7$ pairs. Liver fat discordant group, $n = 8$ pairs. Lfat, liver fat; ApoB, apolipoprotein B; LDL-C, low density lipoprotein cholesterol; IDL-C, intermediate density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol.

of these rare MZ pairs were highly discordant for liver fat, whereas the other half was composed of co-twins which in spite of large differences in body weight had equally low liver fat values in both twin pair members. Using this unique experiment of nature, we demonstrated that the heavier co-twins with high liver fat had higher concentrations of pro-atherogenic lipoprotein particles and less atheroprotective qualities in their lipid profile. The heavier co-twins with low liver fat resembled their lean co-twins more; however, a non-significant trend for worsening of the lipid profile with overall fatness was observed in them as well. Our data further showed that the serum lipid profile is very similar in MZ twins concordant for body weight and remains highly correlated between co-twins in case of TC, IDL-C, HDL-C, and HDL subclasses even in the BMI-discordant pairs. However, TG, ApoB, LDL-C, HDL2a, VLDL, and LDL masses reacted to obesity as the within-pair similarity was lost in BMI-discordant pairs. This suggests that the lipoprotein metabolism has a strong genetic background but there is an interaction with acquired obesity in a complex manner, depending on the distribution of body fat.

The heterogeneity in the lipid panel in our BMI-discordant twin pairs supports previous data on the hypothesis that there might be a distinctive group of metabolically healthy obese people (27). Uncomplicated obesity is characterized by low amount of visceral adipose tissue (28), low inflammatory state (29), and a lower degree of ectopic fat deposition, in particular in muscle and liver (27). Because liver fat and ia fat are highly correlated, their independent roles on the lipid derangements have been difficult to distinguish. This study suggests that liver fat is the main culprit of the lipid disturbances as it was the only body fat depot that remained significant in explaining the lipid parameters in the multivariate models. This finding is in line with the close biological role of the liver as the factory of lipoprotein particles (30).

Fatty liver leads to overproduction of VLDL particles. In addition to overproduction of these TG-rich lipoproteins, hypertriglyceridemia is due to impaired clearance of VLDL particles (7). Overproduction of VLDL has effects both on HDL and LDL via actions of cholesterol ester transfer protein (CETP) secreted in liver. CETP mediates the bidirectional transfer of lipoprotein core lipids between different particles. When VLDL concentration is high, HDL cholesterol esters are preferentially transferred by CETP to larger VLDL particles that become cholesterol rich and thus potentially more atherogenic (31). In addition, CETP is involved with the formation of small LDL particles. This most atherogenic subclass of LDL develops when TGs in LDL are gradually hydrolyzed by hepatic lipase resulting in the formation of small LDL particles (32). Again, our findings showing a pro-atherogenic lipid pattern mainly when the liver is fatty, corresponds well with this biology.

Our data with detailed measures of lipoprotein particles, their size and composition and apolipoproteins have revealed several new findings beyond the traditional measures of basic lipid values. Both LDL-C and LDL particle mass were higher in the heavier co-twins suggesting that the heavier co-twins have an increased number of LDL particles as reflected by the increase of ApoB concentration. In general, HDL is divided into HDL2 and HDL3 subspecies based on their size. While the larger HDL2 has better cholesterol efflux capacity, the smaller and denser HDL3 has been demonstrated to protect LDL from oxidation (33). In this study, ApoA1, the predominant protein carried on HDL particles, did not differ between heavy and lean co-twins. As there was no difference in HDL mass either, it suggests that HDL particle concentration remains unchanged in obesity. Low HDL-C in heavier co-twins was due to a reduction of cholesterol in large HDL2 particles, HDL2b in particular, the subclass that is considered the most cardioprotective (34). Subsequently, HDL particle size was smaller in the heavier co-twins of the BMI-discordant pairs in our data.

Accumulation of fat in the liver has proven to be in part genetically controlled: familial clustering of fatty liver has been demonstrated (35). Approximately 60% of the variability of liver enzymes is heritable (36), and several novel gene candidates and single nucleotide polymorphisms in DNA have been described for explaining either fatty liver (35,37) or liver enzymes (38). It is therefore probable that the propensity to accumulate fat in the liver in obesity is also partly genetic. Notably, in our study, the liver was fatty after development of obesity but not in lean twins, which points to the importance of obesity as the trigger of the genetic predisposition. However, despite the fact that *PNPLA3* and *LYPLAL1* gene variants are significantly associated with liver fat, they do not affect serum lipids (37). This suggests that other underlying mechanisms are involved in the lipoprotein derangements. Whether the same sets of genes explain liver fat and serum lipids remains to be studied.

It is also possible that environmental factors, such as physical activity and diet modify the function of relevant genes and explain the acquired differences in liver fat content and the lipid profile. Exercise training has been proven to reduce intra-hepatic fat content in adults (39). The associations with exercise and lipid levels are less consistent. In Sullivan's trial, no improvement in VLDL-TG or ApoB100 secretion rates was seen (39). In a meta-analysis of randomized controlled-trials in adults improvement was seen only when exercise was combined with diet (40). It is therefore important that we utilized the information from 3-day food diaries and physical activity questionnaires to examine the roles of lifestyle factors on the lipid profile. Low physical activity, together with high liver fat remained as an independent predictor of high LDL-C. In this study, within-pair differences in 3-day food intake or alcohol did not explain the observed lipid changes.

The limitations of our study include the cross-sectional design and the small sample size. It must be acknowledged that this may have limited our power to detect significant differences in the liver fat concordant twin pairs. Furthermore, the possibility of residual confounding by unmeasured covariates cannot be excluded.

In summary, we demonstrate the diversity of serum lipid profile in a unique collection of MZ twins discordant for BMI. Among the BMI-discordant twin pairs, the occurrence of both liver fat discordant and liver fat concordant pairs offered an extraordinary informative design to study the role of body fat distribution and ectopic fat on serum lipids. We were able to show that acquired obesity was associated with a pro-atherogenic lipid profile, in particular when obesity was accompanied by high liver fat. Of note was that despite the probable strong impact of genes on the liver fat and lipid profile, lean individuals remained to a large extent protected from both the fattening of the liver and from the harmful lipid patterns. Physical activity together with low liver fat was the most important cardioprotective factors in this study.O

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Abdominal obesity and circulating metabolites: A twin study approach

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ABSTRACT

Objective. To investigate how obesity, insulin resistance and low-grade inflammation link to circulating metabolites, and whether the connections are due to genetic or environmental factors.

Subjects and methods. Circulating serum metabolites were determined by proton NMR spectroscopy. Data from 1368 (531 monozygotic (MZ) and 837 dizygotic (DZ)) twins were used for bivariate twin modeling to derive the genetic (r_g) and environmental (r_e) correlations between waist circumference (WC) and serum metabolites. Detailed examination of the associations between fat distribution (DEXA) and metabolic health (HOMA-IR, CRP) was performed among 286 twins including 33 BMI-discordant MZ pairs (intrapair BMI difference ≥3 kg/m^2).

Results. Fat, especially in the abdominal area (i.e. WC, android fat % and android to gynoid fat ratio), together with HOMA-IR and CRP correlated significantly with an atherogenic lipoprotein profile, higher levels of branched-chain (BCAA) and aromatic amino acids, higher levels of glycoprotein, and a more saturated fatty acid profile. In contrast, a higher proportion of gynoid to total fat associated with a favorable metabolite profile. There was a significant genetic overlap between WC and several metabolites, most strongly with phenylalanine (r_g = 0.40), glycoprotein (r_g = 0.37), serum triglycerides (r_g = 0.36), BCAAs (r_g = 0.30–0.40), HDL

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particle diameter (r_g = −0.33) and HDL cholesterol (r_g = −0.30). The effect of acquired obesity within the discordant MZ pairs was particularly strong for atherogenic lipoproteins.

Conclusions. A wide range of unfavorable alterations in the serum metabolome was associated with abdominal obesity, insulin resistance and low-grade inflammation. Twin modeling and obesity-discordant twin analysis suggest that these associations are partly explained by shared genes but also reflect mechanisms independent of genetic liability.

1. Introduction

Obesity is often accompanied by a cluster of metabolic abnormalities including insulin resistance, atherogenic dyslipidemia and chronic low-grade inflammation. Although body mass index (BMI) predicts the incidence of diabetes and coronary heart disease [1], it cannot by itself identify obese individuals who remain metabolically healthy and normal weight individuals who present disturbed lipid or glucose metabolism and increased cardiovascular risk [2,3]. The assessment of body fat distribution can further improve the evaluation of the subject's metabolic risk. Indeed, observational studies have shown that abdominal obesity, especially excess visceral [4,5], together with liver fat [3] accumulation is the main driver of cardiometabolic risk factors and disease independently of BMI. Traditionally, serum lipids, glucose and insulin were used as markers of cardiovascular risk. More recently, the focus has widened to cover a more global serum metabolomics profile, which has predicted the incidence of cardiovascular events [6], type 2 diabetes [7] and all-cause mortality [8] in prospective cohort studies.

Both obesity and serum metabolomics profile are heritable. Genetic factors explain 47%–90% of the interindividual variation in BMI with the remaining variance being attributable to environmental sources and measurement error [9]. Heritability estimates from direct measures of whole-body and regional body fat assessed by dual-energy X-ray absorptiometry (DEXA) are generally similar to the estimates obtained for BMI [10,11]. Genetic and environmental influences on serum metabolite levels were recently described in the Finnish twin cohort. Heritability estimates were moderate and ranged between 23% and 55% for amino acids and other small-molecule metabolites and were higher for serum lipid (range: 48%–62%) and lipoprotein (range: 50%–76%) concentrations demonstrating a genetic basis for individual differences in serum metabolite levels [12].

Given these high heritabilities and the significant association between adiposity and metabolic traits [13], a question of interest is whether associations result from potential causal mechanisms or are confounded by shared genes acting pleiotropically on both phenotypes. A recently published Mendelian randomization study suggests causal adverse effects of adiposity with multiple cardiometabolic risk markers in adolescents and young adults from four population-based cohorts in Finland [13]. Obesity discordant twin analyses and bivariate twin modeling are also well suited to explore the extent to which genetic and potential causal environmental factors explain observed associations. Previous twin studies have documented a moderate overlap of both genetic and unique environmental factors that contribute to adiposity and lipid traits [14,15]. However, to the best of our knowledge, they have not yet been extended to include a more comprehensive set of circulating metabolites. Thus, in this study of healthy twins, we aimed to 1) estimate the extent of genetic and environmental overlap between waist circumference (WC) and the serum metabolome using bivariate twin modeling techniques; 2) investigate which adiposity and insulin resistance measures are most strongly associated with the serum metabolic profile, including lipids, fatty acids (FAs), and amino acids in twin individuals; 3) examine whether these associations are independent of genetic and familial influences, by conducting within-pair analysis in monozygotic (MZ) twins.

2. Research Design and Methods

2.1. The Twin Cohorts

The sample was derived from two population-based cohorts, FinnTwin16 (FT16) and FinnTwin12 (FT12) [16]. Both are longitudinal studies of behavioral development and health habits of Finnish twins enrolled during adolescence and repeatedly assessed by self-report questionnaires. The FT12 study includes five consecutive birth cohorts of Finnish twins born in 1983–1987. The questionnaires were sent to twin individuals at age 12 and subsequent follow-up assessments were made when the twins were aged 14, 17 and as young adults (mean age 22 years). The FT16 study includes five consecutive birth cohorts of Finnish twins born in 1975–1979. The questionnaires were sent to the twin individuals at age 16 and subsequent follow-up assessments were made when the twins were aged 17, 18.5, ~25 and ~34 years. For both the FT12 and FT16, the baseline and follow-up assessments included surveys of health-related behaviors, anthropometric characteristics, symptom checklists, and social relationships. Zygosity was determined initially by a validated questionnaire method and then confirmed by genetic analysis of polymorphic markers at the Paternity Testing unit, National Institute for Health and Welfare, Helsinki, Finland.

The data presented in this article were derived from a clinical assessment for twins selected from both FT12 and FT16 after the fourth wave questionnaire collection. Pregnant women and subjects on cholesterol-lowering drugs were excluded. Our study population for quantitative genetic analysis included 1368 subjects (FT12: n = 725, FT16: n = 543 and TwinFat, see below: n = 100; MZ: 531, DZ: 837; age range: 21.0–31.5; 52.5% female). A venous blood sample for serum metabolite (NMR) analyses was taken in the morning of the assessment. Height was measured to the nearest millimeter

with a calibrated stadiometer without shoes and weight to the nearest 100 g with a calibrated scale wearing light indoor clothing. WC was measured to the nearest millimeter midway between the spina iliaca superior and the lower rib.

2.2. *TwinFat — the Sub-sample With Detailed Adiposity Measures*

The TwinFat sample was enrolled from the FT12 and FT16 cohorts based on the twins' BMI at the fourth wave of the data collection. Twins were selected with the aim of covering the full BMI range of both normal-weight and obese subjects and a full range of within-pair differences in BMI. In addition, 100 twin individuals were chosen at random with respect to BMI (and included in the quantitative genetics analysis sample described above). The TwinFat subsample consisted of 286 subjects (MZ: 136 DZ: 150; age range: 22.8–36.2; 52.9% female). 33 MZ twin pairs were discordant for BMI (within-pair difference (Δ) in body mass index ≥3 kg/m^2; age range: 22.8–36.1; 64% female). One twin had type 2 diabetes and used metformin and insulin. Another obese co-twin had inactive ulcerative colitis and used mesalazine and azathioprine. All other participants were healthy (based on medical history, clinical examination, and structured psychiatric interview), were normotensive, and did not use any medications except oral contraceptives. Their weights had been stable for at least 3 months prior to the study. Zygosity was confirmed by genotyping of ten informative genetic markers [14].

Body composition was measured by DXA (Lunar Prodigy, Madison, WI, software version 8.8) providing both total body data and regional results (android, gynoid, arms, legs). Fat percentage was calculated as fat mass/(fat mass + lean mass + bone mineral content) for the total body and android and gynoid fat mass and fat percentage were determined from a total body scan as described by Wiklund et al. [17]. Venous blood samples were drawn after a 12-h overnight fast for the measurement of serum metabolites (NMR), glucose (measured using the spectrophotometric hexokinase and glucose-6-phosphate dehydrogenase; Roche Diagnostics, Basel, Switzerland), serum insulin (time-resolved immunofluorometric assay; Perkin Elmer, Waltham, MA, USA) and high sensitivity C-reactive protein (CRP) (Cobas CRP (Latex)HS, Roche Diagnostics). HOMA-IR was calculated as glucose*insulin/22.5 [18].

A subsample of the TwinFat, 84 MZ subjects (TwinFat Intensive, age range: 22.8–36.2, 52% female) provided magnetic resonance imaging (MRI) measurements for subcutaneous (sc) and intra-abdominal (ia) fat volumes and liver fat content by proton magnetic resonance spectroscopy (MRS) as previously described [19].

Data collection and analysis were approved by the ethics committee of the Department of Public Health of the University of Helsinki, the Institutional Review Board (IRB) of Indiana University and the Helsinki University Central Hospital. All subjects provided written informed consent.

2.3. *The NMR Metabolomics Platform*

All serum samples were analyzed using the same high-throughput NMR metabolomics platform. The sample preparation and NMR spectroscopy methods have been described in detail elsewhere [20,21]. The NMR metabolomics methodology provides quantitative information on lipoprotein subclass and particle concentrations, serum FAs including, e.g. omega-3 and omega-6 FAs and low-molecular-weight metabolites such as amino acids, 3-hydroxybutyrate, and glycoprotein. The total number of metabolites analyzed in the current study was 56. These measures describe the main metabolic pathways. A small number of subjects (<2%) have missing values for some metabolites due to rejection of these values by automatic sample and measurement quality control.

2.4. *Statistical Analyses*

Descriptive characteristics of the study samples were expressed as mean ± SE. Anthropometric measures and serum metabolite concentrations were standardized by age, sex and cohort and rank-transformed to normality prior to statistical analysis. At first, we used the entire sample treating twins as individuals while accounting for twin pair clustering by survey methods [22]. Pearson correlations were used to determine correlations between body composition and serum metabolites. In an initial analysis in which variables were only standardized by age and cohort and not by sex, the correlation coefficients were very similar for men and women. The confidence intervals for the phenotypic correlations between WC and serum metabolites in men and women were wide and overlapping for most variables. In addition, the interaction terms between sex and each metabolite in the prediction of WC were tested and not significant for 54 out of the tested 56 metabolites. Based on these results, we decided to present the results for men and women combined in order to increase the statistical power and to simplify the presentation of the results.

Next, we performed within-pair analyses in MZ twins to examine how differences in body composition are related to differences in metabolites between co-twins. Within-pair differences (Δ) of all measures were calculated by subtracting the leaner co-twins' BMI measures from the ones of heavier co-twins', irrespective of the magnitude of the intrapair difference in weight. Likewise, Pearson correlation analyses were performed to examine how Δbody fat distribution is related to Δ in serum metabolites. The non-parametric Wilcoxon signed-rank test for matched samples was used to compare the untransformed metabolic values between BMI-discordant co-twins. Because MZ twins raised together share their genetic material (i.e. are identical at the DNA sequence level) and a common rearing environment, any observed associations between obesity measures and the serum metabolic profile are fully controlled for unmeasured confounding by genetic and shared environmental effects.

In individual-level analysis, 16 principal components, and in within-pair analysis 15 principal components explained more than 95% of the variance in serum metabolite concentrations. This number was used to correct for multiple testing using the Bonferroni method. The statistical analyses were performed using the Stata statistical software (release 11.0; Stata, College Station, TX) and the rank-transformation to normality was conducted in the statistical software R version 2.15.0 (package GenABEL). Quantitative genetic modeling to estimate these genetic and environmental variance and

covariance components is standard in twin studies [23] and described in more detail in the electronic Supplementary material (ESM) Methods and ESM Figure.

3. Results

Descriptive characteristics of our study cohorts are shown in Table 1.

4. Abdominal Obesity and Serum Metabolites: Bivariate Models

The sex-, age- and cohort-adjusted phenotypic correlations between WC and circulating serum metabolites in individual twins are presented in Fig. 1. The actual Pearson correlation coefficients are shown in ESM Table 1.

4.1. Phenotypic Correlations

WC was significantly correlated with 50 out of the 56 investigated serum metabolites towards an unfavorable metabolic profile. Specifically, abdominal obesity was positively correlated with concentrations of triglycerides and very low-density lipoprotein (VLDL) particles, low-density lipoprotein (LDL) particles, and concentrations of small high-density lipoprotein (HDL) particles, as well as total cholesterol, LDL-C, intermediate density lipoprotein cholesterol (IDL-C), Apolipoprotein (Apo) B and the ApoB to ApoA1 ratio. Concentrations of large HDL particles were negatively correlated with WC. Serum FAs as a proportion of total FAs were inversely, albeit weakly correlated with WC, except for the positive correlation with monounsaturated fatty acids (MUFAs). Branched chain amino acids (BCAA; valine, leucine, isoleucine), two aromatic amino acids (phenylalanine and tyrosine), as well as alanine were positively and glycine negatively correlated with WC. Among the measures of glycolysis, WC was positively correlated with pyruvate and negatively correlated with citrate. WC also correlated positively with glycerol, which serves as a marker of lipolysis of the triglycerides. The ketone bodies acetate and 3-hydroxybutyrate were weakly and inversely correlated with WC. WC was not significantly correlated with the following metabolites: histidine, glycine, lactate, acetate, creatinine and urea. The largest positive correlation was seen with the ApoB to ApoA1 ratio (r = 0.35) followed by glycoprotein (r = 0.33) and the largest negative correlation was observed with HDL particle diameter (r = −0.33) followed by the concentration of large HDL particles and the HDL-C to LDL-C ratio (r = −0.31 for both).

Table 1 – Characteristics of the study cohorts.

	FinnTwin	TwinFat	TwinFat MZ
	n = 1368	n = 286	n =136
Age (yrs)	24.3 ± 0.1	28.7 ± 0.2	29.4 ± 0.5
Monozygotic	531	136	136
Dizygotic, same sex	837	150	NA
BMI (kg/m^2)	23.6 ± 0.1	25.4 ± 0.3	26.5 ± 0.6
Waist (cm)	80.9 ± 0.4	87.3 ± 0.9	88.3 ± 1.4
Total fat (%)	NA	29.0 ± 0.8	31.1 ± 1.3
Android fat (%)	NA	35.0 ± 0.9	37.2 ± 1.3
Gynoid fat (%)	NA	29.9 ± 0.8	38.5 ± 1.3
Android/gynoid fat ratio	NA	1.0 ± 0.0	1.0 ± 0.0
Android fat/Total fat%	NA	1.2 ± 0.0	1.2 ± 0.0
Gynoid fat/Total fat%	NA	1.3 ± 0.0	1.3 ± 0.0
Subcutaneous fat (cm^3) [a]	NA	NA	4411.2 ± 271.9
Intra-abdominal fat (cm^3) [a]	NA	NA	1166.5 ± 110.2
Liver fat (%) [a]	NA	NA	2.9 ± 0.6
Glucose (mg/L)	NA	5.1 ± 0.0	5.2 ± 0.1
Insulin (mU/L)	NA	6.2 ± 0.3	6.0 ± 0.4
Homa IR	NA	1.4 ± 0.1	1.4 ± 0.1
hsCRP (mg/L)	NA	1.7 ± 0.2	2.0 ± 0.3

Values are mean ± SE. MZ, monozygotic; NA, not assessed.
[a] n = 84.

4.2. Genetic and Environmental Correlations

To further investigate the relationship between abdominal obesity and the circulating serum metabolites, we decomposed these phenotypic correlations into their genetic and environmental components (Fig. 1, ESM Table 1). The significant genetic and environmental correlations which were observed between WC and many metabolites indicate that the genetic and environmental factors that influence WC overlap partly with those that influence the serum metabolites. However, the strength of the correlations was weak to modest suggesting that only a fraction of the genetic and environmental variation is common to abdominal obesity and the serum metabolites. Overall, we found that genetic and unique environmental correlations were of about the same magnitude as phenotypic correlations for most serum metabolites. Exceptions were the metabolites ApoA1, BCAA and phenylalanine, for which genetic correlations were stronger than unique environmental correlations. In particular, for ApoA1, valine and leucine, genetic correlations were significant (r_g = −0.24, 0.32 and 0.36 respectively) but the unique environmental correlations were smaller and did not reach statistical significance. This indicates that shared genetic factors largely explain the significant associations between abdominal obesity and these serum metabolites.

5. Obesity-related Measures and Serum Metabolites: Detailed Phenotyping in the TwinFat Sub-sample

Sex- and age-adjusted phenotypic correlations between detailed obesity-related measures and serum metabolites in the TwinFat subsample are presented as a heatmap in Fig. 2. Overall (BMI, % body fat) and abdominal obesity (WC), android fat% and android to gynoid ratio correlated positively with an unfavorable lipoprotein profile (i.e. increased VLDL, IDL, LDL and small HDL particle concentrations, IDL- and LDL-C, triglycerides, ApoB and ApoB to ApoA1 ratio and reduced large HDL particle concentration, HDL-C and diameter). The strongest correlations were observed between measures of abdominal obesity and small VLDL particle concentration, HDL diameter and triglycerides ($r \geq 0.4$). Measures of obesity were not significantly correlated with saturated fatty acids (SFAs) but positively with MUFAs (r_{max} = 0.45) and negatively

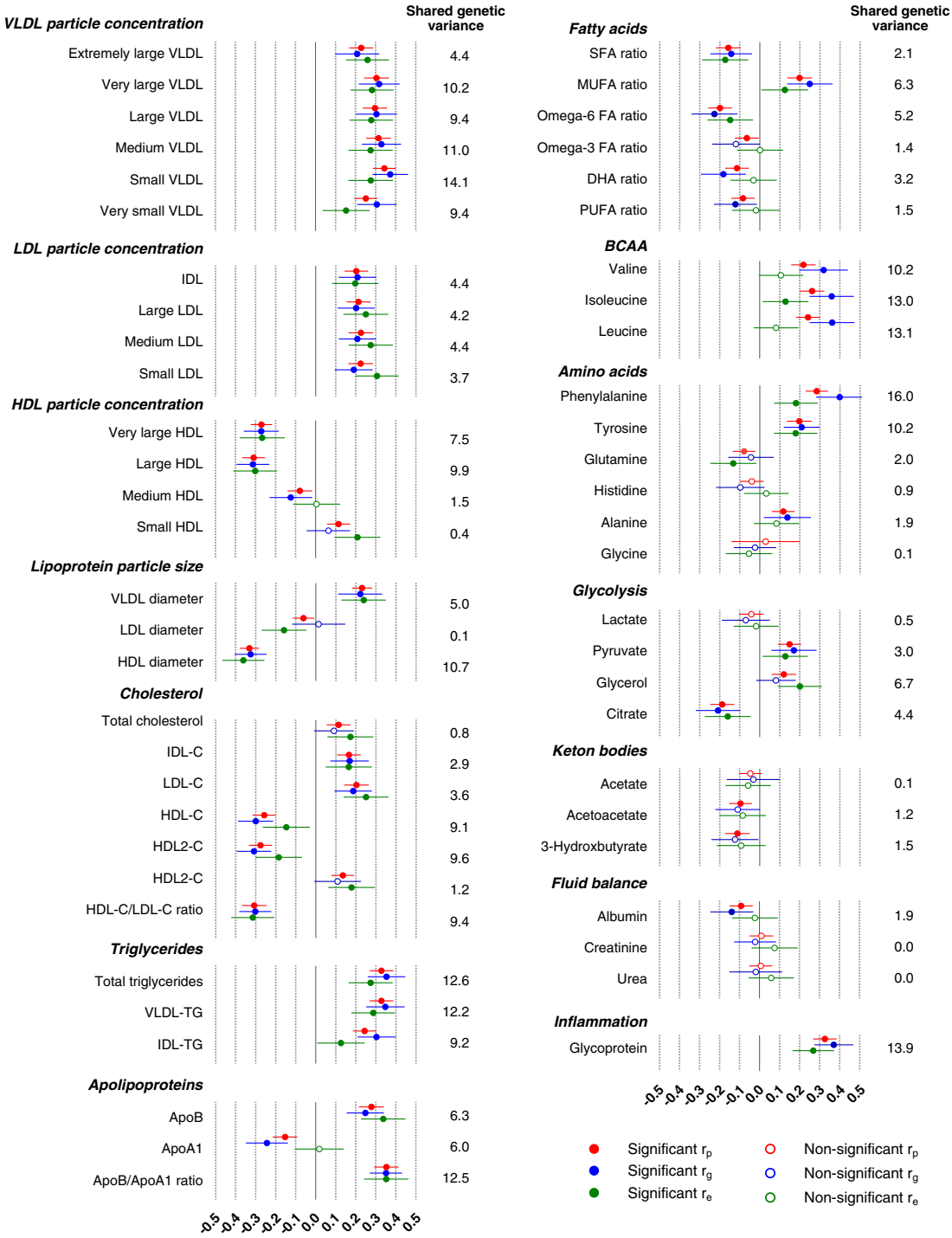

Fig. 1 – Correlations between waist circumference and serum metabolites in 1368 twin individuals. Phenotypic correlations (r_p) are shown in red; genetic correlations (r_g) in blue and unique environmental correlations (r_e) in green. The points indicate the correlation coefficient and the lines show the 95% confidence intervals. All correlations are adjusted for sex, age and cohort. The shared genetic variance was calculated as the r_g^2. Abbreviations: VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; IDL, intermediate-density-lipoprotein; HDL, high-density-lipoprotein; C, cholesterol; ApoB, Apolipoprotein B; ApoA1, Apolipoprotein A1; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; FA, fatty acids; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acids. The fatty acid ratios indicate the ratio of different classes of fatty acids to total fatty acids.

with polyunsaturated fatty acids (PUFAs) ($r_{max} = -0.56$). Obesity was positively correlated with BCAA and phenylalanine ($r_{max} = 0.44$, $p < 0.001$), glycoprotein ($r_{max} = 0.47$) and to a weaker extent with tyrosine ($r_{max} = 0.27$). BMI and measures of abdominal obesity correlated inversely with glutamine ($r_{max} = -0.26$). Measures of insulin resistance (i.e. fasting

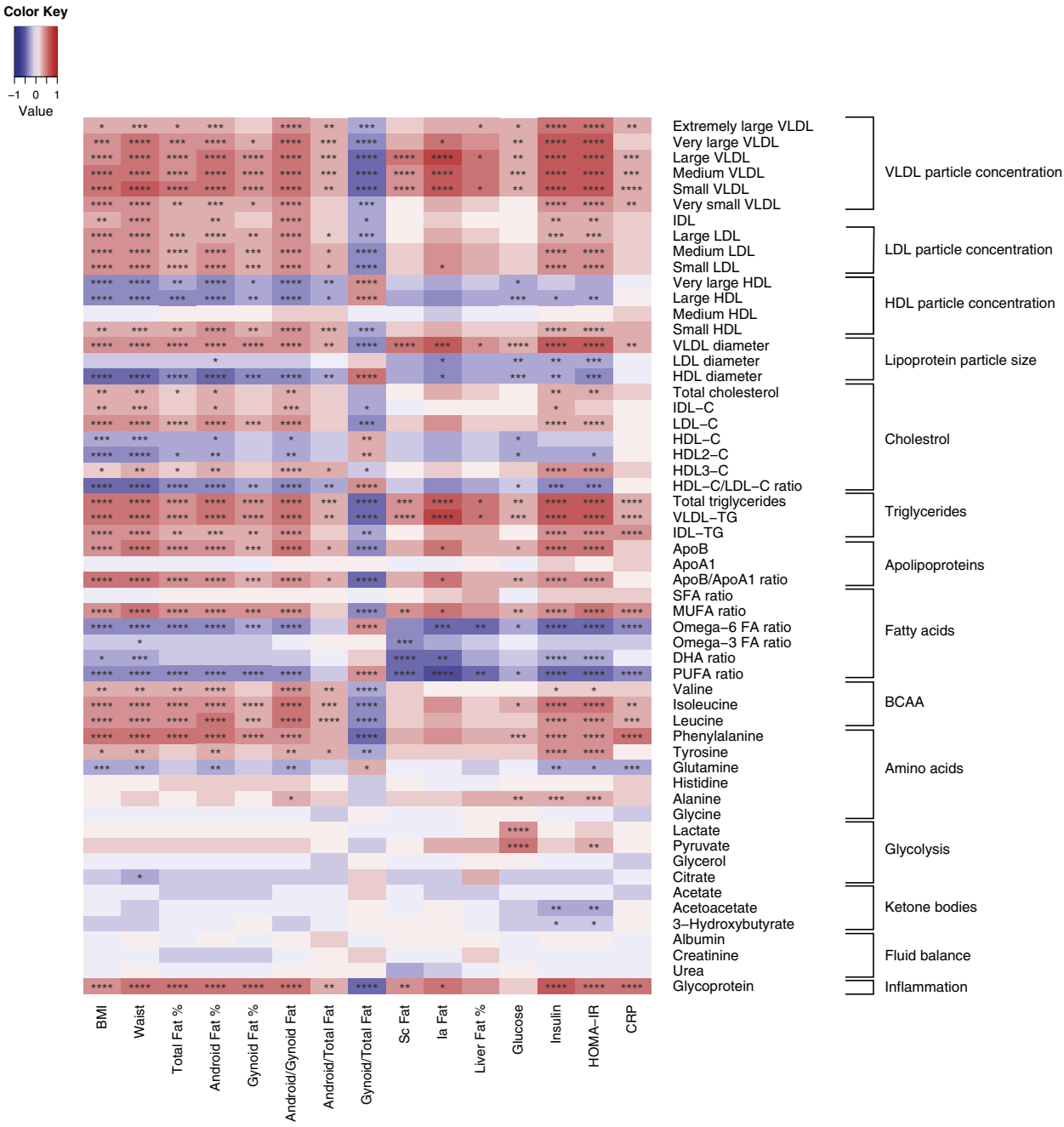

Fig. 2 – Pearson correlations between obesity-related measures and serum metabolites in 286 twin individuals. The sample size for subcutaneous (sc), intra-abdominal (ia) and liver fat was 84. Abbreviations: BMI, body mass index, Waist, waist circumference; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; CRP, high-sensitive C-reactive protein; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; IDL, intermediate-density-lipoprotein; HDL, high-density-lipoprotein; C, cholesterol; ApoB, Apolipoprotein B; ApoA1, Apolipoprotein A1; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; FA, fatty acids; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acids; BCAA, branched-chain amino acids. The fatty acid ratios indicate the ratio of different classes of fatty acids to total fatty acids. Android fat and gynoid fat indicate the ratio of fat to total tissue in the android/gynoid areas. The color key denotes the magnitude of the correlation coefficients. All metabolites were rank-transformed and adjusted for sex and age. The P-values denote the statistical significance after correcting for multiple testing: P-values ****p < 0.00000625; ***p < 0.0000625; **p < 0.000625, *p < 0.0031.

insulin and HOMA-IR) showed similar associations to the metabolites as overall and abdominal obesity although many correlations were stronger (e.g. medium VLDL: r = 0.58, triglycerides: r = 0.56 and glycoprotein: r = 0.48–0.50). Additionally, significant inverse correlations were seen between measures of insulin resistance and LDL diameter, acetoacetate and 3-hydroxybutyrate. CRP correlated with an unfavorable metabolic profile and correlations were generally

weaker than with measures of obesity and insulin resistance, except for the considerable correlation between CRP and glycoprotein (r = 0.48). The associations between the ratio of gynoid to total fat mass and the metabolic profile were opposite than with other obesity measures. Correlations were strongest with HDL diameter (r = 0.41) and the following metabolites: medium and small VLDL particles, triglycerides, phenylalanine and glycoprotein (r = −0.43 to −0.48).

The same analysis that was performed in individual twins was repeated using within-pair differences (Δ) in MZ twin pairs, thus being able to control for familial and genetic confounding. Compared with analyses performed in twin individuals, there was a notable drop in the strength of most correlation coefficients (Fig. 3). This indicates possible genetic control over the association between obesity measures, insulin resistance, inflammation and the serum metabolic profile. However, exceptions were the following: Δandroid fat% correlated with lipoprotein profile including ΔLDL-C (r = 0.46), ΔLDL particle concentrations (r_{max} = 0.49), ΔLDL diameter (r = −0.41) and ΔApoB (r = 0.47). Similarly, Δinsulin resistance associated with an unfavorable metabolic profile including ΔVLDL particle concentration (r_{max} = 0.56), Δtriglycerides (r = 0.53), ΔPUFAs (r = −0.41), Δisoleucine (r = 0.46) and Δglutamine (r = −0.43). The correlations between Δia fat and Δliver fat and ΔLDL particle concentrations, ΔLDL-C, ΔApoB, ΔApoB to ApoA1 ratio, ΔHDL diameter, Δacetoacetate, and Δ3-hydroxybutyrate were stronger than in individual-level analysis. The highest correlation was seen for correlations between Δia fat and Δliver fat and the ΔApoB to ApoA1 ratio (r = 0.61 and 0.58, respectively). This highlights that acquired accumulation of fat in the abdominal area, and excess ia and liver fat in particular, are important modifiable environmental factors that modify the levels of atherogenic lipoproteins and some other metabolites.

5.1. *BMI-discordant Monozygotic Co-twins*

We further studied the role of environmental factors by comparing the serum metabolites between 33 BMI-discordant MZ twin pairs. The obese co-twins were on average 18 kg heavier and had 29% more total fat, 36% more fat in the android and 17% more fat in the gynoid area and 280% more liver fat. In obese co-twins we indeed found significantly increased levels of those metabolites that correlated strongest with obesity measures above (ESM Table 2). Obese co-twins had higher concentrations of VLDL and LDL particles, cholesterol, triglycerides, ApoB, BCAA, lactate, glycerol, urea and glycoprotein than their leaner counterparts. Obese co-twins had lower concentrations of HDL particles, HDL-C and a smaller HDL diameter. The ratios HDL to LDL, ApoB to ApoA1 and PUFA to total FAs were lower in the obese than lean co-twins. The particle concentration of most lipoproteins, HDL particle size and ratios of HDL to LDL and ApoB to ApoA1 remained significantly different between co-twins even after correction for multiple comparison (adjusted $p < 0.0033$).

6. Discussion

In this study of healthy young adults, abdominal obesity, insulin resistance and low-grade inflammation were associated with higher concentrations of atherogenic lipoproteins, aromatic and BCAA, glycoprotein and lower concentrations of PUFAs and glutamine. The relative contribution of genetic and modifiable environmental factors in explaining these associations was dependent on the metabolite under investigation. Acquired obesity, independent of genetic factors within the MZ BMI-discordant twin pairs, was most clearly associated with an atherogenic lipoprotein profile.

Previous twin studies have reported that genetic correlations are stronger between physiologically similar phenotypes, such as triglycerides and HDL-C, and weaker for other pairs of phenotypes, such as obesity and blood pressure [24,25] or obesity and lipid traits [15,26]. In the present analysis, we extend these earlier observations by quantifying the genetic and environmental factors that underlie abdominal obesity and a large number of metabolites, which are potential novel biomarkers of metabolic diseases. Genetic correlations between abdominal obesity and metabolites were highest for HDL-C and diameter, serum triglycerides, the ApoB to Apoa1 ratio, BCAA, phenylalanine and glycoprotein. Future bivariate linkage and genome-wide association studies are needed to co-localize these correlated traits to a genetic region. As an evidence of a common genetic basis between obesity and serum lipids, previous bivariate linkage studies have reported a pleiotropic quantitative trait locus (QTL) on chromosome 19 which jointly influences serum triglycerides and adiposity [27] and an LDL-C–BMI locus on chromosome 3 [28]. He and colleagues [29] found an inverse association between a genetic risk score for BMI and HDL-C in type 2 diabetic women, which was independent of BMI, indicating that pleiotropic genes could influence both phenotypes through different pathways. Kilpeläinen et al. [30] reported that a locus near IRS is associated with lower body fat percentage and, opposite to what would be expected, with higher insulin resistance and an adverse lipid profile.

Detailed phenotyping of obesity-related measures in a subsample of the twins allowed the investigation of which obesity-related measures were most closely linked to an adverse metabolic risk profile. As previously reported for standard lipid measures [17,31], abdominal obesity and insulin resistance were the strongest correlates of an atherogenic lipoprotein profile including triglycerides and triglycerides in VLDL and IDL, LDL-C, IDL-C and ApoB, VLDL and LDL particle subclasses of all sizes as well as small HDL particle subclasses. Furthermore, obesity correlated negatively with concentrations of large HDL subclasses, HDL-C and HDL-C to LDL-C ratio. This increase in total VLDL and LDL concentration and shift in the lipoprotein subclass distribution towards larger VLDL and smaller HDL and LDL particles are consistent with increased cardiovascular disease risk [32].

In the present study, correlations within MZ twin pairs were generally weaker than in individual-level analysis. This provides evidence of partial confounding due to genetic factors, which is consistent with the finding of significant genetic correlations for abdominal obesity and most metabolites in the quantitative genetic modeling. It is also in agreement with results from large GWAS studies that identified loci and genes associated with these traits [33]. However, measures of abdominal fat distribution (i.e. android fat% and ia fat), liver fat and insulin resistance were more

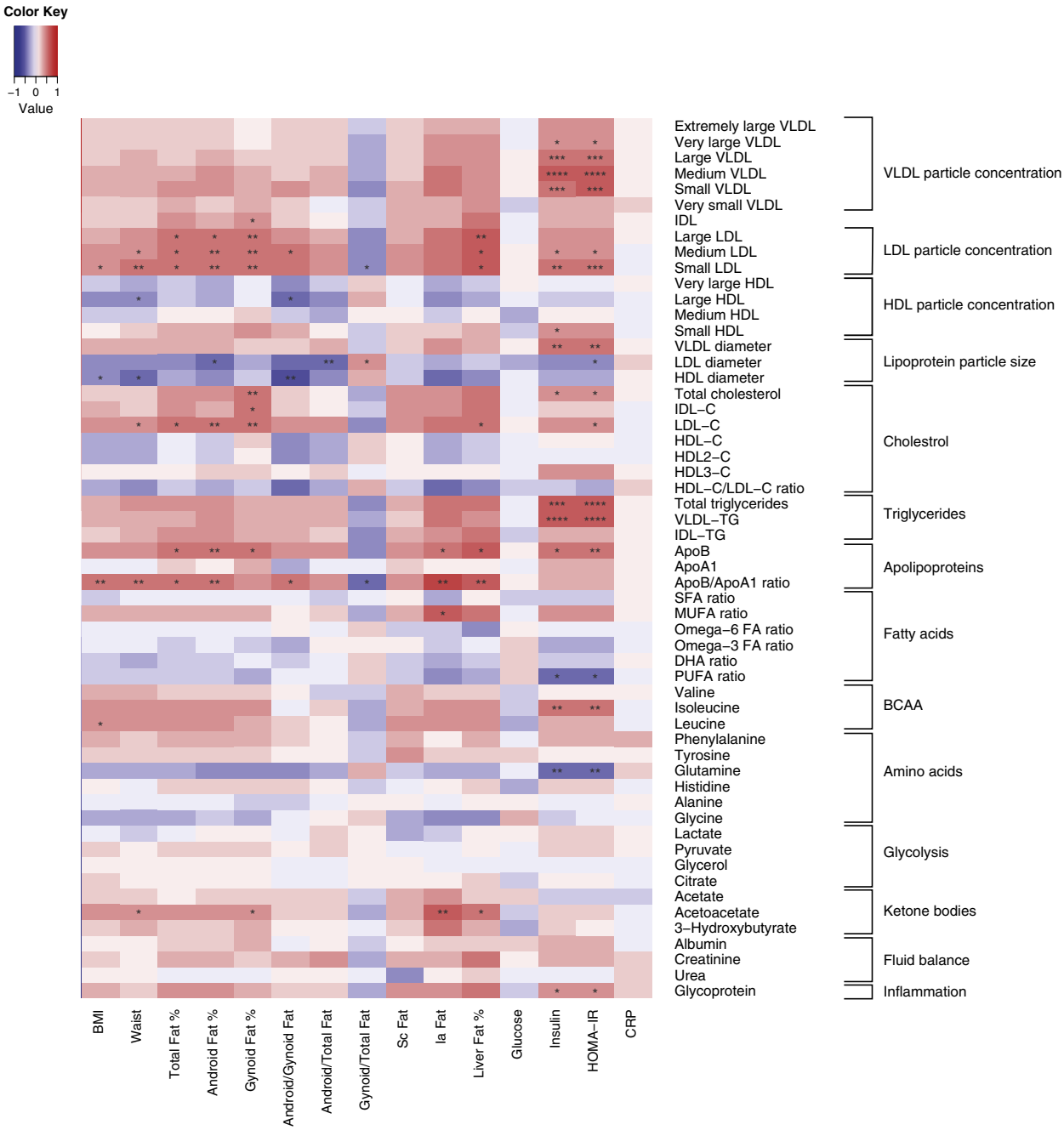

Fig. 3 – Pearson correlations between within-pair differences in obesity-related measures and within-pair differences in serum metabolites in 68 monozygotic twin pairs. The sample size for subcutaneous (sc), intra-abdominal (ia) and liver fat was 42 pairs. Abbreviations: BMI, body mass index, Waist, waist circumference; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; CRP, high-sensitive C-reactive protein; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; IDL, intermediate-density-lipoprotein; HDL, high-density-lipoprotein; C, cholesterol; ApoB, Apolipoprotein B; ApoA1, Apolipoprotein A1; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; FA, fatty acids; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acids; BCAA, branched-chain amino acids. The fatty acid ratios indicate the ratio of different classes of fatty acids to total fatty acids. Android fat and gynoid fat indicate the ratio of fat to total tissue in the android/gynoid areas. The color key denotes the magnitude of the correlation coefficients. All metabolites were rank-transformed and adjusted for sex and age. The P-values denote the statistical significance after correcting for multiple testing: P-values ****p < 0.000006667; ***p < 0.00006667; **p < 0.0006667, *p < 0.0033.

strongly related to VLDL particle concentration, ApoB, LDL-C and LDL diameter in within-pair than in individual-level analysis. Similarly, the heavier co-twins of MZ twin pairs had 280% more liver fat and a more atherogenic lipoprotein profile even after correcting for multiple testing. The well-known physiologic role of liver in the lipoprotein metabolism

suggests that liver fat in our twins is causally linked to the higher VLDL, LDL, and ApoB concentrations. In accordance, lipoprotein kinetic studies in humans have shown that liver fat drives the overproduction of large VLDL particles and ApoB, which in turn initiates a number of lipoprotein changes that increase the risk of atherosclerosis, including hypertriglyceridemia, an increase in small, dense LDL particles and low HDL-C [34].

We found that obesity and insulin resistance were associated with increased concentrations of MUFAs and lower concentrations of PUFAs, in particular omega-6 FAs and to a lesser extend omega-3 FAs. The serum FA profile is influenced by dietary fat intake over the past few weeks [35], and we have previously reported a lower proportional dietary PUFA intake in the heavier co-twins of the discordant MZ pairs [36]. Rate-limiting enzymes are also important determinants of the serum FA profile; in particular stearoyl-CoA desaturase-1 (SCD-1) synthesizes MUFA from SFA and D6-desaturases synthesizes highly unsaturated FAs. Subjects with obesity or metabolic syndrome have increased activity of SCD-1 and D6-desaturase [37], which corresponds well with our finding of more MUFAs and less PUFAs in the serum of obese subjects. In the present study, fasting insulin and HOMA-IR remained significantly correlated with lower concentrations of PUFAs in analysis that controlled for potential confounding by familial factors of genes and shared environment within MZ twins. In two Finnish studies, low concentrations of serum omega-6 PUFAs were prospectively associated with the incidence of impaired fasting glycemia, diabetes mellitus [38] and metabolic syndrome [39].

In our healthy twin sample of young adults, abdominal obesity and insulin resistance were the strongest correlates of BCAA, phenylalanine and tyrosine. It has been known for many years that obesity is associated with an increase in these amino acids [7,40]. More recently, it has been shown that obesity blunts amino acid metabolism in adipose tissue and profoundly affects BCAA catabolism. In subcutaneous and visceral adipose tissue obtained during surgery, essential amino acids, leucine, glutamine and serine and 2-ketoisocaproic acid differed significantly between obese and lean subjects [41]. In our earlier study of MZ twin pairs discordant for obesity, serum levels of insulin secretion-enhancing BCAA were increased in obese male co-twins and adipose tissue transcription profiles exposed significant down-regulation of genes involved with mitochondrial BCAA catabolism [3,42] indicating that the increase in plasma BCAA levels may be due to their decreased oxidation in the peripheral tissues. Our finding of an inverse association between glutamine and insulin resistance is in accordance with previously published data from the Framingham Heart Study and the Malmö Diet and Cancer Study [43]. In the present study we highlight the complexity in the association between obesity and amino acid metabolism, which involves both genetic and environmental factors.

Measures of obesity, insulin resistance and CRP correlated with glycoprotein, an acute-phase protein that is elevated in response to inflammation and infection [44]. Alpha-1 acid glycoprotein has been prospectively associated with both fasting and postchallenge glucose [45] and with the risk of developing diabetes [46]. In a recent study among participants from the Estonian biobank, alpha-1 acid glycoprotein was the strongest circulating biomarker predicting all-cause mortality after adjusting for conventional risk factors [8].

Among all obesity measures studied, measures of abdominal obesity and the ratio of android to gynoid fat were the strongest correlates of an unfavorable serum metabolic profile. In contrast, correlations with the ratio of gynoid to total fat mass were in the opposite direction, supporting the view that having proportionally more fat stored in the gynoid area is metabolically protective. It has been suggested that gluteofemoral fat functions as a 'metabolic sink' to buffer excess energy, thereby preventing ectopic and visceral fat deposition [47].

Combining men and women in the analysis can be seen as a potential limitation. However, we did not observe significant differences by sex in the associations of obesity and metabolites, and therefore combined men and women in the analysis in order to increase statistical power. A previous large study of obesity and circulating metabolites that included 12,664 adolescents and young adults from four population-based cohorts in Finland found significant sex interactions for some metabolites; however the absolute differences were fairly small [13]. The strengths of the present study include its population-based design, the detailed phenotyping of body composition, the inclusion of large sets of serum metabolites and the use of twins to tease out genetic and environmental factors. Potential shortcomings of the study must also be acknowledged. First, the young adult and Caucasian sample utilized in this study limits the generalization to other populations. Second, the cross-sectional study setting does not provide understanding of the temporal relations, even though the use of the MZ discordant pairs permits control of genetic and early family confounders. Finally, multiple comparison correction and the reduced sample size in MZ within-pair and discordant twin analysis increase the likelihood of false negatives (type II error).

In conclusion, abdominal obesity and insulin resistance are associated with changes in the serum metabolome toward reduced cardiometabolic health. Our results suggest that genes that influence variation in abdominal obesity partly overlap with those that influence variation in serum metabolites. However, genetically identical twins that differ in obesity measures also differ in the serum lipoprotein profile, therefore indicating that obesity may be a modifiable environmental factor that leads to lipid disturbances. Thus, our findings give further credence to public health efforts aiming to prevent weight gain and closely linked metabolic abnormalities.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.metabol.2015.10.027.

Author Contributions

JK, KHP and AR collected the data and recruited study subjects. LHB and SK researched data and wrote the manuscript. JR and AOA assisted in statistical analyses. PS, AJK and MAK performed NMR analyses. AH, JL and NL measured and interpreted the MRI and MRS data. All authors reviewed and edited the manuscript and approved the final version of the manuscript. KHP is the guarantor of this work and, as such,

had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Conflict of Interest

AJK, PS and MAK are shareholders of Brainshake Ltd, a startup company offering NMR-based metabolite profiling.

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Upregulation of Early and Downregulation of Terminal Pathway Complement Genes in Subcutaneous Adipose Tissue and Adipocytes in Acquired Obesity

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Inflammation is an important mediator of obesity-related complications such as the metabolic syndrome but its causes and mechanisms are unknown. As the complement system is a key mediator of inflammation, we studied whether it is activated in acquired obesity in subcutaneous adipose tissue (AT) and isolated adipocytes. We used a special study design of genetically matched controls of lean and heavy groups, rare monozygotic twin pairs discordant for body mass index (BMI) [$n = 26$, within-pair difference (Δ) in body mass index, BMI >3 kg/m^2] with as much as 18 kg mean Δweight. Additionally, 14 BMI-concordant (BMI <3 kg/m^2) served as a reference group. The detailed measurements included body composition (DEXA), fat distribution (MRI), glucose, insulin, adipokines, C3a and SC5b-9 levels, and the expression of complement and insulin signaling pathway-related genes in AT and adipocytes. In both AT and isolated adipocytes, the classical and alternative pathway genes were upregulated, and the terminal pathway genes downregulated in the heavier co-twins of the BMI-discordant pairs. The upregulated genes included C1q, C1s, C2, ficolin-1, factor H, receptors for C3a and C5a (C5aR1), and the iC3b receptor (CR3). While the terminal pathway components C5 and C6 were downregulated, its inhibitor clusterin was upregulated. Complement gene upregulation in AT and adipocytes correlated positively with adiposity and hyperinsulinemia and negatively with the expression of insulin signaling-related genes. Plasma C3a, but not SC5b-9, levels were elevated in the heavier co-twins. There were no differences between the co-twins in BMI-concordant pairs. Obesity is associated with increased expression

of the early, but not late, complement pathway components and of key receptors. The twins with acquired obesity have therefore an inflated inflammatory activity in the AT. The results suggest that complement is likely involved in orchestrating clearance of apoptotic debris and inflammation in the AT.

Keywords: obesity, complement system, gene expression, twin study, monozygotic twins

INTRODUCTION

The complement system has a pivotal role in obesity. While it is an important innate immune defense system against microbes and part of the body's clearance system, it can also regulate the level of inflammation in the adipose tissue (AT) and have metabolic effects. Complement is a key innate sensor between viable and non-viable cells. It recognizes and opsonizes both foreign targets, like microbes, and exposed or damaged endogenous structures, and promotes their clearance by macrophages. Altogether, the complement system comprises up to 50 proteins that are synthesized by several tissues including the liver and various cell types of AT (adipocytes, macrophages, and vascular cells) (1–3). The complement proteins circulate in blood as activating or regulating components or act on cell membranes as receptors or protective molecules. Intriguingly, many links exist between the complement system and AT, but overall, the meaning and relevance of these links are mostly unclear.

The complement system can be activated through the classical, the lectin, or the alternative pathway. Specifically, the classical and the lectin pathways can recognize targets with specific pattern recognition molecules C1q, mannan-binding-lectin (MBL), and ficolins 1–3 (FCN1–3) (1–3). In contrast, the alternative pathway is continuously active and can amplify efficiently complement activation against non-self targets (2–4). All three pathways merge to activate the complement component C3, the most abundant complement factor in human blood plasma. Factor D (CFD, adipsin) is synthesized solely by adipocytes and its plasma levels are decreased in obesity (5). Factor D activates complement factor B (CFB) allowing the generation of the alternative pathway C3 convertase C3bBb, which enzymatically cleaves new C3 molecules to C3a and C3b (1–3). Opsonization by C3b and by its inactivated product iC3b leads to the phagocytosis of target structures (1, 3). Activation of the early pathways of the complement system results in the generation of C5a, a potent anaphylatoxin, chemoattractant, and leukocyte activator. Together with C3a, C5a can attract cytokine-producing macrophages and other leukocytes to the area of microbial invasion or tissue damage (1, 3). The completion of the cascade, the terminal pathway, leads to formation of the membrane attack complex (MAC, C5b-9) that allows sodium and calcium influx into cells. Membrane leakage will thus sequentially lead to strong metabolic activation, injury, and ultimately death of the target cell (4). Soluble complement inhibitors such as factor H (CFH), vitronectin and clusterin, and a group of membrane-bound regulators [complement receptor 1 (CR1)/CD35, CRIg, MCP/CD46, DAF/CD55, and protectin/CD59] protect normal viable human cells against complement attack (3).

The complement system includes many proteins relevant to obesity, e.g., acylation-stimulating protein (ASP, C3a desArg) and adipsin (factor D). In addition, the activation-initiating complement proteins are related to adiponectin, which is an insulin-sensitizing and anti-inflammatory adipokine. Its plasma levels decrease in obesity (6). Adiponectin has structural similarity to complement collectins C1q, MBL, surfactant proteins SP-A and SP-D, and FCN1–3. They are all multimers of triple-helical structures, where each subchain has subunits composed of a globular and a collagen-like domain (7). Additionally, adiponectin participates in the clearance of dying cell material by macrophages via a calreticulin-mediated mechanism (8) and is an immune-modulator lowering tumor-necrosis factor-alpha (TNF-α)-levels (9). ASP is identical to the C3 activation product C3a desArg (10). In AT, ASP stimulates glucose uptake and free fatty acid storage postprandially (10). In the complement system, C3a desArg is mostly devoid of the anaphylatoxin or chemotactic activity of C3a. While C3a binds to the C3a receptor (C3aR), C3a desArg binds to the C5a receptor type 2 (C5aR2), which often mediates anti-inflammatory effects (11). C3aR expression has been shown to become upregulated in AT following a high-fat diet (12). It is intriguing that the C3a-C3aR (inflammation) and C3a desArg-C5aR2 (fat and glucose metabolism) interactions have such different effects.

During the progression of obesity, the adipocyte size increases, which associates with inflammation (13) and is linked to the development of insulin resistance (14, 15). Plasma levels of C3, CFH, and CFB correlate positively with body mass index (BMI), waist circumference, triglycerides, and inflammatory parameters and negatively with insulin sensitivity and HDL cholesterol (16, 17). Complement components such as C3 and factor B are overproduced by activated adipocytes in type 2 diabetes mellitus (T2DM) (18), and C3 is among the major determinants of metabolic syndrome in obese patients (19). Thus, the activated complement system is involved in the pathogenesis of cardiovascular disease (20). Upregulated complement gene expression by adipocytes from subcutaneous (sc) fat associates with insulin resistance (21, 22). In a population-based cohort study, plasma levels of C3 were found to correlate positively with plasma insulin and glucose and to associate with the development of type 2 diabetes (23). Since complement is a main mediator of inflammation and clearance of non-viable tissue components, its role in obesity-related fat overload and metabolic disturbances merits more attention.

In humans, most of the studies on the complement system have been conducted in unrelated individuals without control for genetic variation. It is evident that genetic effects have a strong influence on both how complement activation is linked to inflammation (24) and on the weight gain (25) as well as for the developing complications thereafter. To exclude confounding due to shared genetic factors, we undertook a unique approach of analyzing complement gene expression in subcutaneous AT of genetically identical twins either concordant or discordant for obesity. To exclude the effect of the immune-cell-rich

stroma-vascular fraction, we further analyzed the complement gene-expression profiles of isolated adipocytes. In addition to comparing the co-twins (effects of acquired obesity), we wanted to know whether complement gene expression is associated with adipocyte size, fat distribution (subcutaneous, intra-abdominal, and liver fat), systemic inflammation, systemic markers of insulin resistance, or the expression of insulin-signaling genes. This was explored separately in the AT and isolated adipocytes from the twin individuals. Finally, we stained AT biopsies immunohistochemically to investigate the localization and intensity of C1q, the key activator of the classical pathway and of C3d, an indicator of prolonged complement activation. The results reveal an unprecedented coupling of the complement recognition molecules and early activation pathways with acquired obesity and related metabolic disturbances.

MATERIALS AND METHODS

Participants

The study population has been described in our previous studies (26–28). Briefly, the monozygotic (MZ) twin pairs (aged 22–36, 54% women) were identified through the national population registry of Finland from 10 population-based birth cohorts (FinnTwin12 and FinnTwin16); 26 pairs being discordant for BMI, within-pair difference (Δ) in BMI >3 kg/m^2 (mean Δweight 18 kg), and 14 pairs concordant for BMI (ΔBMI <3 kg/m^2) acting as their controls. All pairs were of European ancestry (Finnish), normotensive, and did not use any other medications than oral contraceptives, except for one obese co-twin with T2DM who used metformin and insulin, and one obese co-twin with inactive ulcerative colitis who used mesalazine and azathioprine. Twenty-three subjects were habitual smokers. AT biopsies were available from all 80 subjects. mRNA was available for gene expression analyses in isolated adipocytes from 38 subjects (14 BMI-discordant and 5 concordant pairs). The clinical characteristics within these twin pairs did not differ from the entire group of 80 twins (Table S1 in Supplementary Material). The protocol was designed according to the principles of the Helsinki Declaration and all subjects gave their written informed consent. The Ethical Committee of the Helsinki University Central Hospital (DNRO 270/13/03/01/2008) approved the protocol.

Serum and Plasma Analyses, Body Composition, AT Biopsies, and Adipocyte Morphology

Venous blood samples were obtained from all subjects after overnight fasting. EDTA plasma and serum samples were separated by centrifugation and stored at −80°C until the analyses of plasma glucose (spectrophotometric hexokinase and glucose-6-phosphate dehydrogenase assay, Roche Diagnostics, Basel, Switzerland), serum insulin (by time-resolved immunofluorometric assay; Perkin Elmer, Waltham, MA, USA), plasma adiponectin and adipsin (DuoSet ELISA, R&D Systems Europe Ltd., Abingdon, UK), and serum high-sensitive C-reactive protein [hsCRP, Cobas CRP (Latex) HS, Roche Diagnostics] were performed. Plasma levels of fluid phase activation products of the complement system C3a (cleaved from C3) and SC5b-9 (soluble C5b-9) were determined by an enzyme-linked immunosorbent assay using MicroVue C3a Plus and C5b-9 Plus enzyme immunoassay kits (Quidel Corporation, San Diego, CA, USA) according to the manufacturer's instructions. The absorbances were read at 450 nm in a FluorStar Optima multidetection microplate reader (BMG Labtech GmbH, Ortenberg, Germany), and the concentrations analyzed using Optima's MARS 2.0 analysis software.

Body composition of the subjects was measured by Dual-energy X-ray absorptiometry (Lunar Prodigy, Madison, WI, USA, software version 8.8). Fat percentage was calculated as fat mass/(fat mass + lean mass + bone mineral content) for the total body. Magnetic resonance imaging (MRI) was used for measuring the volumes of abdominal subcutaneous and intra-abdominal fat, and magnetic resonance spectroscopy for measuring the liver fat percentage, as described earlier (29).

Adipose tissue biopsies were obtained from periumbilical subcutaneous fat using a surgical technique. RNA was prepared from frozen fat tissue (26). Adipocytes were isolated from the fat biopsy specimens treated with collagenase (28). The diameter of fat cells was measured under a light microscope. Fat-cell volume was calculated as described by Heinonen (13) assuming the adipocytes as spheres.

Transcriptomics Analyses of AT and Adipocytes

Transcriptomics experiments were performed with Affymetrix U133 Plus 2.0 chips. Raw gene expression data were preprocessed with the GeneChip robust multiarray averaging (GCRMA) algorithm using BioConductor (30) in R and by using Brainarray custom CDF files for probe annotations (31). The data were validated with RT-qPCR as described (32). We selected 46 representative genes coding for components of the complement system for the expression analyses in the AT (Table S2 in Supplementary Material). The following genes from the insulin signaling pathway were used in analyses of the relationships between complement system and insulin sensitivity in AT: insulin receptor (INSR), insulin-receptor substrate proteins 1–2 (IRS1–2), and phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA). The following complement factors were not included as they were not included in the array (C1QTNF4, C1QTNF6, C4, MBL, MASP-2) or were expressed at low levels (C8, C9, C4BP, C5aR2, and vitronectin) thereby lacking the inter-individual variation. GLUT4 gene was expressed at the lowest detection level, lacked inter-individual variation, and was therefore excluded from AT analyses.

Isolated adipocyte transcriptomics experiments were performed with Affymetrix U133 Plus 2.0 chips similarly as for the AT samples. The expression levels of L-selectin (SELL) and C5aR2 were not found from adipocyte transcripts. The same insulin signaling pathway-related genes than from AT and GLUT4 gene transcripts were analyzed.

Immunohistochemical Staining

We selected two BMI-discordant pairs and two BMI-concordant control twin pairs to visualize complement proteins in AT. Paraffin blocks of the AT were sectioned at 5 μm and two sections were

placed on each slide. One of the sections was used for staining with a primary and secondary antibody, while the other served as a mock control with buffer pipetted in place of the primary antibody.

First, a standard de-paraffination procedure was applied consisting of 2× xylene (5′), followed by a series of ETOH (2′ each) in decreasing concentration and ending up in distilled water where samples slides were stored until the following day in preparation for staining. An antigen retrieval heat-treatment protocol followed, where section slides in EDTA buffer pH8.5 from Sigma (product# E1161, Sigma-Aldrich, St. Louis, MO, USA) + 0.05% Tween were brought to a boiling point in a microwave oven for 15′ followed by cooling in RT for 30′. The actual staining was carried out using 3× PBS washed heat-treated slides in a humidity chamber using Bright Vision plus Poly-HRP-Anti MS/RB/RT IgG REF DPVB55-HRP kit (Immunologic, Duiven, Netherlands). The grease pen limited section was subjected for Hydrogen Peroxide Block (UltraVision; Thermo Fisher Scientific, Waltham, MA, USA) incubation for 10′, where after the manufacturer's protocol for the staining kit was followed. The primary antibodies were diluted 1:1,000 in PBS (C1q and C3d both Rabbit pAb by DAKO, Glostrup, Denmark). The final step of the staining protocol was counterstaining by Gills Hematoxylin (2″) rinsing (10′) and finally series of ETOH (2′) treatments in increasing concentration finishing in 2× xylene (2′) and mounting by Depex. The slides were dried ON in a fume hood and stored in RT. Tissue histology was confirmed by standard HE staining for each tissue sample. Images of immunohistochemistry were collected using Olympus DP Manager (ver. 2.2.1.195) and Olympus DP Controller (ver. 2.2.1.227) image capture softwares with Olympus BX51 fluorescence microscope camera with 100×, 200×, and 400× magnifications.

Statistical Analyses

The statistical analyses were performed using Stata statistical software (release 13.0; Stata Corporation). In addition to controlling the effect of gender, the comparison of MZ co-twins (almost 100% identical at the DNA sequence level) allows controlling for genetic and shared environmental effects. Thus, any differences within twin pairs are by definition acquired (in our case, through BMI difference). Comparisons of the heavier vs. leaner co-twins' clinical characteristics were analyzed by paired *t*-test (normally distributed) or Wilcoxon's test (non-normally distributed variables). Within-pair comparisons of the $\log^2$-transformed gene expression values were tested by paired *t*-test.

Student's *t*-test was used for the co-twin comparison of the gene expression data. In addition, we assessed differences between genders, and smokers and non-smokers (individual twins) with Mann–Whitney *U*-test. Since some differences in the gene expression between smokers vs. non-smokers emerged, within-pair analyses were performed first among all twin pairs, whereafter habitual smokers were removed from the leaner vs. heavier comparison to assess whether the effect of smoking was confounding the results. As the differences in the gene expression between leaner and heavier co-twins still emerged to the same extent before and after exclusion of the smokers, we present the results from the whole cohort, with smokers included. Within-pair analyses are by design adjusted for gender.

Mann–Whitney *U*-test was used to compare whether the characteristics of the leaner and the heavier co-twins in the BMI-discordant pairs were the same in the whole group for whom AT was available and in those for whom data on adipocytes were available.

Using twins as individuals, the partial correlation analyses were performed to determine the relationships between AT/adipocyte gene expression and adiposity measures, adipokines, and the insulin signaling gene expression. Since differences between genders and smokers vs. non-smokers emerged in individual twins' gene expression, their effect was adjusted in correlation analyses. Logarithmic corrections were performed for log-normally distributed variables prior to the correlation analyses. Subsequently, the *p* values of partial correlates were adjusted for multiple testing by false-discovery rate (FDR) (Benjamini–Hochberg) (33).

RESULTS

Study Population

Table 1 summarizes the subjects' anthropometric and clinical characteristics (26–28). In brief, the heavier co-twins of the discordant pairs weighed on average 18 kg more, had 28% more total fat, 67% more subcutaneous fat, 200% more intra-abdominal fat, and 300% more liver fat than their leaner counterparts. Accordingly, the adipocyte volumes of the heavier co-twins were 57% larger than those of the lean co-twins. Furthermore, the heavier co-twins had higher plasma levels of insulin and adipsin, and lower adiponectin levels. BMI-concordant pair co-twins did not differ in any of the metabolic measures. They were also similar for all complement-related measures, which is why we report only the results from the discordant pairs.

The Early Complement Pathway Components Are Upregulated and C3 Is More Activated in Heavier Co-twins

Initially, we analyzed the effect of obesity on complement gene expression. In AT, 20 out of the altogether 46 complement genes expressed differently between the leaner and the heavier co-twins of the BMI-discordant pairs (**Figure 1**; Table S2 in Supplementary Material). In heavier co-twins of BMI-discordant pairs, the C1qA-C genes, those of the classical pathway activation initiating proteins, were upregulated. Accordingly, the genes for other classical pathway components: C1s, the key catalytic component of C1, the receptor for C1q (C1QR), C1r-like protease (C1RL), and C2 were upregulated. In contrast, the genes of FCN2, coding a protein recognizing acetylated carbohydrates in the lectin-pathway, and C1QTNF7, a C1q and TNF-related protein (CTRP) family member, were downregulated in heavier co-twins.

The gene expression of the most abundant complement inhibitor factor H (CFH) of the alternative pathway was upregulated in heavier co-twins compared to their leaner counterparts. Additionally, complement receptor of the immunoglobulin superfamily (CRIg/VSIG4) was upregulated. Thus, while the early classical pathway components were upregulated, the result suggests that complement activation would continue only up to

TABLE 1 | The clinical characteristics of the monozygotic twin pairs.

	BMI-discordant pairs			BMI-concordant pairs (n = 14)	
	ΔBMI > 3 kg/m², n = 26 pairs			ΔBMI < 3 kg/m², n = 14 pairs	
Age (mean years ± SD)	30.4 ± 4.2				
Sex	9 males/17 females			5 males/9 females	
	Leaner Mean ± SE	**Heavier Mean ± SE**	***p***	**Leaner Mean ± SE**	**Heavier Mean ± SE**
N of current smokers	7	7		4	5
BMI	25.28 ± 0.89	31.25 ± 1.02	<0.0001	26.20 ± 0.92	27.65 ± 1.01
Fat percentage (%)	32.25 ± 1.81	41.14 ± 1.32	<0.0001	28.56 ± 2.57	29.89 ± 2.39
Adipocyte volume (μm³)[a]	371.9 ± 34.05	584.4 ± 49.55	<0.0001	364.7 ± 54.11	403.1 ± 46.87
Subcutaneous fat (cm³)	3,814 ± 416.9	6,359 ± 540.4	<0.0001	3,084 ± 351.7	3,428 ± 394.0
Intra-abdominal fat (cm³)	790.2 ± 179.0	1,644 ± 247.4	<0.0001	1,037 ± 173.2	1,093 ± 200.3
Liver fat%	1.12 ± 0.32	4.52 ± 1.00	<0.0001	1.99 ± 0.90	3.75 ± 1.75
fP-insulin (mU/L)[b]	4.93 ± 0.51	8.50 ± 1.21	<0.001	5.34 ± 1.13	5.61 ± 0.60
fP-glucose (mmol/L)[b]	5.13 ± 0.07	5.28 ± 0.11	0.174	5.27 ± 0.11	5.44 ± 0.15
hsCRP (mg/L)	2.56 ± 0.70	4.02 ± 1.14	0.065	1.25 ± 0.55	1.11 ± 0.19
Adipsin (pg/L)[a]	1,192 ± 49.05	1,309 ± 47.34	0.006	1,011 ± 121.7	1,047 ± 81.97
Adiponectin (ng/L)	3,842 ± 284.9	2,820 ± 232.2	0.0001	3,370 ± 539.9	2,603 ± 307.9
fP-C3a (ng/mL)	69.20 ± 4.16	77.82 ± 4.57	0.016	67.70 ± 9.24	61.45 ± 3.28
fP-SC5b-9 (ng/mL)	184.6 ± 10.28	193.64 ± 9.00	0.209	187.0 ± 15.13	184.3 ± 13.19

Wilcoxon signed-ranks test (leaner vs. heavier twin). Within-pair difference BMI >3 kg/m² in discordant pairs (n = 26 pairs, 9 males), BMI <3 kg/m² in concordant pairs (n = 14 pairs, 5 males).

BMI, body mass index; fP, fasting plasma; hsCRP, high-sensitive C-reactive protein; C3a, complement component 3a; SC5b-9, the soluble terminal complement complex.

[a]n = 8 concordant pairs.

[b]25 discordant pairs.

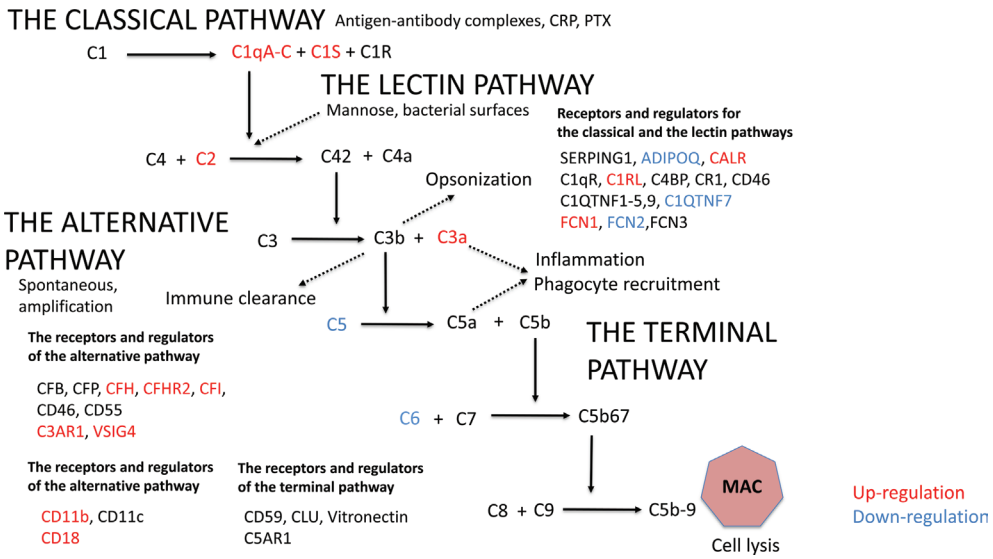

FIGURE 1 | The gene expression profile in subcutaneous adipose tissue within monozygotic body mass index-discordant twin pairs (heavier vs. leaner) differed in 20 out of 46 genes that code for components of the complement system. Genes that are upregulated in heavier co-twins are indicated with red and downregulated genes with blue.

the C3/C5 convertase level because of upregulation of the main inhibitory control factor CFH. In accordance, the plasma levels of C3a and adipsin were higher in the heavier, but the levels of SC5b-9, a marker of terminal pathway activation, were similar between the heavier and the leaner co-twins of BMI-discordant pairs (**Table 1**). This indicates that complement activation in

obese individuals indeed occurred up to the C3 activation level but not beyond that to the terminal pathway.

Upregulated Gene Expression of Complement Receptors and Complement Inhibitors and Downregulation of the Terminal Pathway in AT of Heavier Co-twins

The genes for C3aR1 and C5aR1, important complement receptors found, e.g., on the surfaces of macrophages and other immune cells, were upregulated in heavier co-twins' AT (**Figure 1**; Table S2 in Supplementary Material). Furthermore, the heavier co-twins had an upregulated gene expression of components of the beta-integrin CD11b/CD18, the major phagocytic receptor (CR3) for particles coated with iC3b. The genes of the terminal pathway components C5 and C6 were downregulated, whereas the gene of the soluble terminal complement complex binding clusterin (CLU) was strongly upregulated in heavier co-twins. This indicates that the terminal pathway is suppressed in obese individuals, whereas the receptors for early activation pathway components are upregulated to mediate their functional effects.

Complement Gene Expression Profiles of Isolated Adipocytes Differ between BMI-Discordant Co-twins

As AT is a mixture of adipocytes, immune cells, vasculature, and the connective tissue stroma, we next wanted to study the expression profiles of complement genes in isolated adipocytes. The levels of 25 out of 45 complement genes expressed by isolated adipocytes differed between heavier and leaner co-twins of the BMI-discordant pairs (Table S3 in Supplementary Material). Similar to AT, in the adipocytes, the complement genes of the classical and alternative pathways were upregulated and genes of the terminal pathway were downregulated in heavier co-twins. However, a few differences in adipocytes compared to the AT expression profile were observed: ADIPOQ, FCN1, CALR, and CFH gene expressions were similar between co-twins of BMI-discordant pairs, but the gene expressions of C1-inhibitor (SERPING1), CD93, C3, CFB, C5AR1, and the MAC-inhibiting molecule CD59 (protectin) were upregulated in heavier co-twins. Again, the gene expression profiles of isolated adipocytes of the co-twins of BMI-concordant pairs were similar (data not shown).

Adiposity Measures Associate with Upregulated Expression of Genes in the Classical and the Alternative Pathways and Downregulation of Genes in the Terminal Pathway-Related Genes

The correlations between adiposity measures, metabolism, inflammation, and complement gene expression profiles in AT and adipocyte transcripts are presented as heatmaps in **Figure 2**. Table S4 in Supplementary Material shows the corresponding *r* values and *p* values after correction for multiple parameters.

In AT samples, the complement gene expression levels correlated significantly with BMI (18 out of 46 genes), adipocyte volume (23/46), sc fat (22/46), intra-abdominal fat (29/46), liver fat (22/47), hsCRP (28/46), and plasma adipsin (18/46) levels. The correlations of adiposity measures were positive for genes of the classical and the alternative pathway components and negative for the terminal pathway components C5 and C6. Furthermore, SERPING1 and the C1q homologs C1QTNF7 and FCN2 correlated negatively with adiposity measures.

The complement gene expression of isolated adipocytes correlated most significantly with intra-abdominal fat (24 out of 45 genes), sc and liver fat (13/45 for both), and hsCRP (23/45). The correlations were positive except for C1QTNF7, FCN2, C5, and C6.

Upregulation of the Classical and Alternative Pathway Components Associates with Hyperinsulinemia and Downregulated Insulin Signaling Route

Next, we examined whether the complement gene expression in AT and adipocytes was associated with plasma insulin and expression of genes along the insulin signaling route (INSR, IRS1–2, and PIK3CA in both AT and adipocytes, and additionally GLUT4 in adipocytes) (**Figure 2**; Table S4 in Supplementary Material for the corresponding *r* values and *p* values after correction for multiple parameters). A strong inverse correlation between the classical and the alternative pathway and insulin signaling route gene expression was found both in AT and adipocytes. Plasma insulin correlated positively with the expression of 25/46 complement genes in AT but only with CR1 gene expression of adipocytes. C6, C1QTNF7, FCN2, and FCN3 expression in AT correlated negatively with plasma insulin.

Visualization of Complement Proteins in AT

Finally, we investigated the location of two complement proteins by immunohistochemistry from paraffin-embedded AT samples. Two complement components were stained: C1q indicating initiation of the classical pathway and C3d representing the remnant of C3b deposition typically observed in prolonged complement activation (examples shown in **Figures 3A–D**). Crown-like structures representing tissue macrophages around apoptotic adipocytes were observed in both leaner and heavier co-twins of the BMI-discordant pairs, even though they seemed to be more abundant in heavier co-twins' AT. C1q stained abundantly on cell membranes and intracellularly in the apoptotic cells. Occasionally, intense nuclear staining of adipocytes occurred adjacent but independently of the crown-like structures. C3d stained faintly but evenly on the cell membranes – most likely on the basal surface of the stain-positive cells. Additionally, intracellular granular aggregates staining intensively for C3d emerged in the crown-like structures, but also in some individual adipocytes.

DISCUSSION

The present study illustrates how obesity can induce a coordinated and synchronized regulation of the complement system genes.

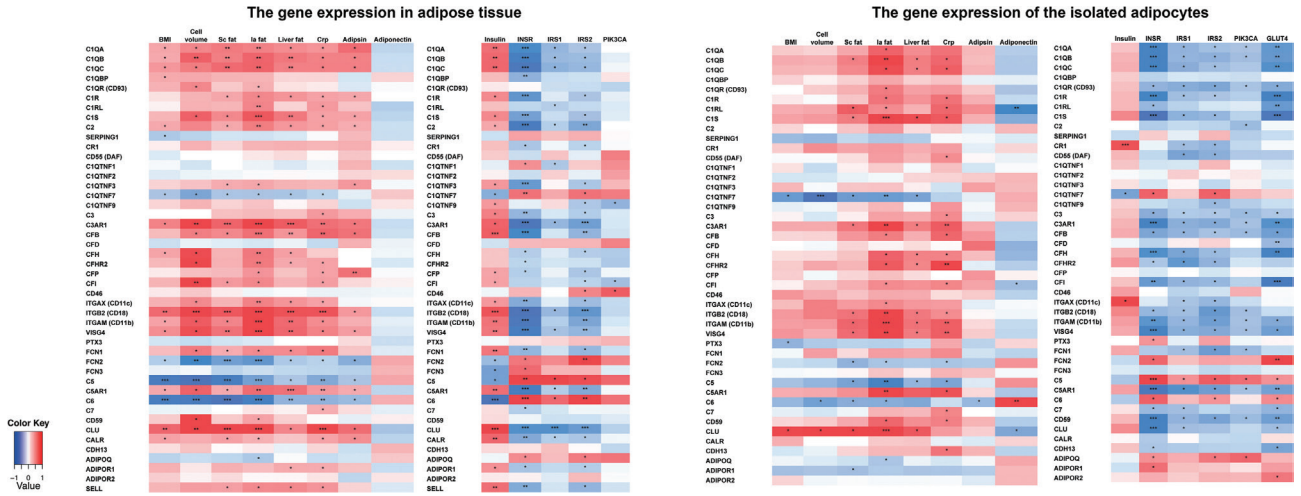

FIGURE 2 | The correlations between the complement gene expression and the obesity measures, inflammation, and insulin signaling-related genes in monozygotic (MZ) twin individuals taking into account the effect of gender and smoking. MZ twin individuals, $n = 80$; cell volume, $n = 65$; plasma adipsin, $n = 70$; plasma adiponectin, $n = 80$; insulin, $n = 80$; adipocyte gene transcripts, $n = 38$. *Statistical significance, $p < 0.05$, **$p < 0.001$, ***$p < 0.0001$ after multiple correction (false-discovery rate, Benjamini–Hochberg). BMI, Body mass index; Cell volume, adipocyte volume; Sc fat, subcutaneous fat; Ia fat, intra-abdominal fat; Liver fat, liver fat percentage; CRP, high sensitive C-reactive protein; INSR, insulin receptor; IRS1-2, insulin receptor substrate 1-2; PIK3CA, Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Alpha; GLUT4, glucose transporter type 4.

The expression of a broad spectrum of complement genes was analyzed in AT and isolated adipocytes in young adult MZ twins, nearly all of whom were free from obesity-related co-morbidities. Within BMI-concordant pairs, the co-twins' gene-expression profiles were similar suggesting that the overall expression levels of complement genes are largely familial, and probably controlled by genetic or shared environmental factors.

Several interesting differences emerged within BMI-discordant MZ twins: the expression of genes of complement activating and regulating components within AT and isolated adipocytes was clearly different in heavier compared with their leaner co-twins. Most of the differences in the co-twins remained in isolated adipocytes' transcripts even after separating them from the immune cell-rich stroma-vascular fraction. The main findings were the following: in heavier co-twins, the classical and the alternative pathway complement gene expressions were mostly upregulated. These pathways mediate all main physiological functions of the complement system. They recognize microbes, antigen–antibody complexes, materials from injured cells and tissues, and other targets. Complement activation products promote opsonization, chemotaxis, leukocyte recruitment, activation, and phagocytosis. These effects are mediated by specific receptors that, accordingly, were also upregulated. In line with these results, the plasma levels of C3a, a marker of the activation of early complement pathways, were elevated in the heavier co-twins. Instead, the levels of the soluble terminal pathway complement complex SC5b-9 remained unchanged. This matches with the gene expression data, where the terminal pathway components C5 and C6 were downregulated, while the inhibitor clusterin was upregulated. When combined, the results demonstrate that complement is involved in obesity-related inflammation but not in direct MAC-mediated tissue destruction.

Our results demonstrating the effects of excess body weight in young adults are in accordance with previous studies reporting upregulated complement gene-expression levels in AT in obese, dyslipidemic, and diabetic subjects (4, 21, 23). We made several interesting new observations on upregulated complement genes in the heavier co-twins' AT and on their association with obesity and metabolic disturbances. All the three sub-chains of C1q, A-C, were upregulated in obesity. They constitute C1q, a multimeric protein that binds to antigen–antibody complexes and to many types of cellular structures, which are either exposed or released during cellular damage (phospholipids, mitochondria, etc.). Bound C1q is subsequently recognized by macrophage receptors that mediate phagocytosis of the targets (34, 35). A novel observation was that the C1r-related protein C1RL gene expression increases in acquired obesity and associates with accumulation of intra-abdominal fat. C1QTNF7, a member of the CTRP family and closely related to C1q, was downregulated in heavier co-twins. Interestingly, C1QTNF7 responds to caloric restriction in mice and therefore has a potential role in the control of the energy balance (36). Furthermore, calreticulin, a C1QR, showed upregulated gene expression in the heavier co-twins and correlated positively with hyperinsulinemia and negatively with the genes involved in the insulin signaling route. The results from Jalali (37) showing the association between INSR density and calreticulin, and Lo (5) demonstrating that calreticulin correlates positively with pancreatic insulin secretion increasing adipsin, support a potential role for calreticulin in the regulation of glucose metabolism.

Another novel observation was that genes of the C3 convertase inhibitor CFH and the alternative pathway suppressing VSIG4 (CRIg), a member of the complement receptor immunoglobulin superfamily, were upregulated in heavier co-twins. VSIG4

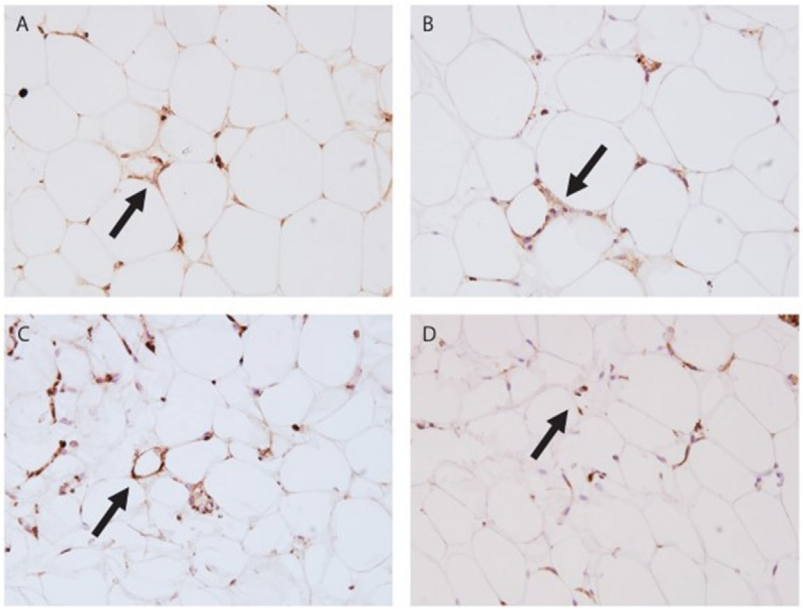

FIGURE 3 | **Immunohistochemistry stain of subcutaneous adipose tissue (AT)**. **(A)** Leaner twin AT stained with C1q (400×); **(B)** leaner twin AT stained with C3d (400×); **(C)** heavier twin AT stained with C1q (400×); **(D)** heavier twin AT stained with C3d (400×); the arrows point out crown-like structures.

correlated strongly positively with overall and intra-abdominal fat and with plasma insulin levels. VSIG4 inhibits T-cell proliferation, clears complement-opsonized particles, and binds to C3b (38). VSIG4 is known as a potential biomarker of severe preeclampsia (39). Therefore, despite the upregulation of complement activating components C3 and CFB in obese subjects, the concomitant upregulation of the alternative pathway regulatory genes indicates that an excessive activation and amplification of this pathway can be suppressed in AT. Alternative pathway activation occurs in a regulated manner generating C3a, and possibly C5a (by the C3/C5 convertases), as well as the C3b inactivation product iC3b (by CFH and CFI), but remains at a reasonable level to prevent transmission of amplification to the terminal pathway.

The levels of SC5b-9, a soluble equivalent of MAC with only one C9 remained unchanged in the heavier co-twins. In the heavier co-twins, upregulation of complement inhibitors and downregulation of the expression of C5 and C6 indicate that excessive early pathway activation in obesity does not translate into the terminal pathway. Thus, complement activation is related more to increased opsonophagocytic and anaphylatoxic capacity than to direct cell damaging activity by MAC. Clusterin gene expression showed overall the most significant differences within BMI-discordant co-twins and the strongest positive correlation between obesity measures, liver fat, hsCRP, hyperinsulinemia, and adipsin and negative correlation with insulin signaling route gene expression in twin individuals. Our results are in line with Won et al. (40), who showed that subjects with metabolic syndrome have elevated levels of clusterin in plasma. In fact, clusterin is a multifunctional molecule, whose activities extend beyond those in the complement system. In addition to being an inhibitor of MAC, it can cluster cells and act as an apolipoprotein, hence the alternative names SP40, 40, and ApoJ (41).

Clusterin suppresses the terminal pathway by inhibiting formation of the complement MAC, composed of components C5b, C6, C7, C8, and multiple C9 molecules (42). Clusterin prevents insertion of intermediate terminal pathway complexes (C5b-7, C5b-8, and C5b-9) to membranes. During this process, it incorporates into the complexes. Increased expression of clusterin may thus be related to the inhibition of excess complement-mediated cell damage that is initiated by increased expression of early pathway components, ischemia, or the release of complement-activating lipids from adipocytes. In addition, by being an apolipoprotein and scavenger for hydrophobic molecules, clusterin could also directly bind to hydrophobic particles released from fat tissue and play a role in their removal.

Adipocyte cell volume was associated with complement gene expression in the AT similarly as the overall obesity measures. A positive association emerged for the adipocyte size with the classical and the alternative pathway and a negative one with the terminal pathway-related genes. The adipocytes undergo changes in their cell membranes in the development of the obese state and potentially this alters the structural conformation and type of surface membrane molecules they express (43). Large adipocytes act as antigen-presenting cells by expressing major histocompatibility class II molecules and take part in the generation of pro-inflammatory immune response (43). Contrasting and potentially generating a balancing response, C1q, a structural homolog of adiponectin, promotes anti-inflammatory macrophage polarization (35, 44, 45). Thus, upregulated C1q expression in AT may be reactive and signal a tissue defense mechanism and local immune cell infiltration. It is noteworthy that multiple C1q homologs, adiponectin, FCN1–2, and C1qTNF7 showed changes in obesity. This suggests their involvement in an adipocyte-macrophage collaboration process,

possibly related to debris clearance. As we were limited by sample availability, the immunohistochemical stains did not provide quantifiable data. In the descriptive analyses, a pattern emerged, where C1q stain appeared more intense and widespread in the obese twins than their lean co-twins. The pattern of C1q may reflect ongoing phagocytosis (e.g., of necrotic or apoptotic adipocytes) and/or increased inflammation in the heavier co-twins albeit being present also in the leaner.

The associations between intra-abdominal fat, liver fat, crp, and the genes of the insulin signaling route and differences in most of expression levels of the complement genes remained significant in adipocyte transcripts after the contribution of the stroma-vascular fraction was excluded. In contrast, the role of the stroma may be significant when interpreting the correlations between BMI, sc fat. adipocyte cell size, sc fat and insulin. The correlations between complement gene expressions and these measures were weaker in adipocyte transcripts than in AT. Indeed, it is not the obesity *per se* but the central adiposity and ectopic fat in liver that are related to alterations in the complement gene expression.

Our study of MZ twin pairs is unique in that genetic factors as causes for differences in within-pair comparisons can be excluded. Esparza-Gordillo (24) estimated that 62% of the complement factor H (CFH) phenotypic variation is due to additive genetic effects. Nestvold (46) showed that the complement system is reactive to environmental factors as levels of C3 and C4 dropped after weight loss. The observed differences within weight-discordant pairs emphasize the role of non-genetic influences, including environmental factors and acquired obesity in regulating the expression and activation of the complement system. Finding healthy young adult MZ pairs with significant discordance in body weight is extremely difficult, as body weight is tightly genetically regulated (47). The experiences and exposures of MZ twins are often very similar through childhood and adolescence and begin to differ only after moving out of their parental home and into individual personal and occupational trajectories (48). Therefore, although our sample size is relatively small, the study represents perhaps the best-controlled study design available in humans because of the full match for genes, age, gender, and intrauterine and childhood environment between the lean and heavy groups.

The limitations of our study include that due to small biopsy materials from the volunteers, we lack protein or functional data on the AT samples. However, we documented that circulating C3a protein levels were elevated in obesity, potentially indicating complement activation also at the whole-body level. Our gene-expression analyses also lacked the data for some components of complement cascade such as the pattern recognition protein MBL, C4, C8, and C9. The study setting does not allow direct causal conclusions, but indicates that the observed differences within BMI-discordant co-twins are not due to confounding genetic effects.

In summary, our study shows that characteristic to complement activation in obese AT is upregulation of the classical and the alternative pathway sensors and receptors and downregulation of the terminal pathway. In the AT, the activating factors and the receptors were upregulated simultaneously indicating preparedness for controlled phagocytosis. In addition to the pattern-recognition molecules needed for phagocytic activity, similar gene-expression patterns also emerged in isolated adipocytes, which indicates their independent role in inducing changes in the complement system in the obese AT. Overall, the study reveals the close relationship between the complement system and AT in obesity. These results pave the way for further analyses of the complement-mediated regulation of inflammation and lipid clearance and its potential role leading to adverse metabolic complications in obesity.

ETHICS STATEMENT

The protocol was designed and performed according to the principles of the Helsinki Declaration and was approved by the Ethical Committee of the Helsinki University Central Hospital (DNRO 270/13/03/01/2008).

AUTHOR CONTRIBUTIONS

Contributions of the authors were as follows: KP, JK, and AR collected the data. SM, A.Hanttu, and KP designed the studies. SK, KP, and SH performed the clinical studies. AIL performed the immunohistochemistry analyses, EN measured the plasma complement levels and SH calculated the adipocyte volumes. A.Hakkarainen, NL, and JL performed and assisted in measuring the magnetic resonance imaging-related data. OT processed tissue samples for the study. MM and LS assisted in the bioinformatics. SK, A.Hanttu, and SM analyzed and interpreted the data. SK wrote the first draft of the manuscript. SK and AIL edited the final manuscript after all authors had commented and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00545/full#supplementary-material.

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